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KRISTINA HAHN, Department of Microbiology and Immunology and the Center for Oral Biology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA

DAVID A. HODGSON, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK (dm@dna.bio.warwick.ac.uk)

WENDI L. KUHNERT, Department of Microbiology and Immunology and the Center for Oral Biology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA

ROBERT QUIVEY, Department of Microbiology and Immunology and the Center for Oral Biology, University of Rochester School of Medicine and Dentistry, Box 611, 601 Elmwood Avenue, Rochester, NY 14642, USA (Robert_Quivey@urmc.rochester.edu)

GERALD WILLIAM TANNOCK, Department of Microbiology, University of Otago, PO Box 56, Dunedin, New Zealand (gerald.tannock@stonebow.otago.ac.nz)

CHRISTOPHER F. THURSTON, Division of Life Sciences, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 8WA, UK (christopher.thurston@kcl.ac.uk)

J. R. WHITEFORD, Division of Life Sciences, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 8WA, UK

The Molecular Genetics of Cultivated Mushrooms

J.R. Whiteford and C.F. Thurston

Division of Life Sciences, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 8WA, UK

Dedicated to the late Dr David A. Wood

ABSTRACT

The types, economic significance and methods of production of the principal cultivated mushrooms are described in outline. These organisms are all less than ideal for conventional genetic analysis and breeding, so molecular methods afford a particular opportunity to advance our understanding of their biology and potentially give the prospect of improvement by gene manipulation. The sequences described are limited to those found in GenBank by August 1999. The gene sequences isolated from the white button mushroom *Agaricus bisporus*, the shiitake *Lentinula edodes*, the oyster mushrooms *Pleurotus* spp., the paddy straw mushroom *Volvariella volvacea* and the enotake *Flammulina velutipes* are described. The largest group are genes from *A. bisporus*, which includes 29 for intracellular proteins and 12 for secreted proteins. In comparison, only a total of 26 sequences can be reported for the other cultivated species. *A. bisporus* is also the only cultivated species for which molecular karyotyping is already supported by reliable markers for all 13 of its chromosomes.

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1. INTRODUCTION

1.1. The Principal Cultivated Mushroom Species

Mushroom cultivation is an economically significant and rapidly expanding global industry (Flegg *et al.*, 1985; Chang, 1993). Traditionally, it has been divided between south-east Asia, including China and Japan on the one hand, and Europe, North America and Australasia on the other. The Asian production has been diverse with several/many relatively low-volume crops and a few of high production, dominated by *Lentinula edodes* (Shiitake), *Pleurotus* spp. (the oyster mushroom) and *Volvariella volvacea* (the paddy straw mushroom) (Chang and Miles, 1991). The European and American industry has (until recently) produced *Agaricus bisporus*, the white button mushroom, almost exclusively (Table 1). Although a handful of other mushrooms are cultivated to a significant extent, the major change of the 1980s and 90s was the widespread introduction of the 'Asian' mushrooms into western markets and the very rapid increase in production of *Pleurotus* spp. (Chang, 1993). In Japan there has also been a very large increase in *Flammulina velutipes* (Enokitake), such that its production in that country was comparable to that of *Lentinula edodes* throughout the 1990s (Yamanaka, 1997). Elsewhere in Asia, production of *Auricularia* spp. has increased significantly as well.

Table 1 World mushroom production of principal species (freshweight, megatonnes).[a]

Organism	1986	1991
Agaricus bisporus	1.215	1.590
Lentinula edodes	0.320	0.526
Volvariella volvacea	0.178	0.253
Pleurotus spp.	0.169	0.917
Auricularia spp.	0.119	0.465
Flammulina velutipes	0.100	0.187
'Total'	2.176	4.273

[a]From Chang (1993). The total values include other more minor species. *Lentinullla edodes* was until recently *Lentinus edodes*, but molecular taxonomy has shown that this organism is distinct from other members of the genus *Lentinus*.

Table 2 World cultivated mushroom production: FAO data.[a]

Year	1981	1986	1991	1996	1997	1998
Production (Mtonnes)	1.124	1.409	1.804	2.084	2.095	2.100

[a]Selected years, from http://Apps.FAO.ORG/default.htm.

Up-to-date figures for world mushroom cultivation are uncertain. FAO (the Food and Agriculture Organisation of the United Nations) figures showed a striking increase in total tonnage every year through to 1996, although the most recent data show less increase (Table 2), but these figures (where comparable) are substantially less than those given by Chang and Miles (1991) and Chang (1993); see Tables 1 and 2. Whatever the total may be, mushroom production is a large, expanding and highly competitive industry across many countries.

1.2. Cultivation Methods

The fungi that produce large fleshy fruiting bodies, including an estimated 2000 species that are good to eat (Chang, 1991), are mostly basidiomycetes, the notable exceptions being the Morels and Truffles which are ascomycetes. Most of these species have yet to be cultivated and, in some cases, the mycorrhizal association of the fungus with plant roots makes facile cultivation unlikely (it is frequently a simple matter to grow vegetative mycelium, but producing fruiting bodies is quite another matter). The principal cultivated species are, in contrast, all wood or leaf-litter degraders. *Agaricus bisporus* has a specialized nutritional pattern growing on composted leaf material that can be produced so as to form a very selective medium for this fungus (Flegg *et al.*, 1985). In contrast, *Lentinula edodes* is a wood degrader that has traditionally been grown on logs, but which is increasingly being produced on sawdust based material. Indeed, sawdust and other wood wastes, sometimes mixed with straw or rice bran, are becoming the main production material of all the large-volume mushrooms except *A. bisporus* (Ogha and Kitamoto, 1997; Yamanaka, 1997).

These fungi are all able to degrade the major polymers of woody plant material, lignin, cellulose and the hemicelluloses, although the ability to completely mineralize lignin is not definitely established in all cases. The processes are therefore attractive because agro/industrial wastes are consumed in the production of foods that have both high economic and nutritional value.

1.3. Mushroom Production as Biotechnology: the Uses of Molecular Genetics

The driving forces behind molecular genetic studies of cultivated fungi are three-fold. First, it may be that the mushrooms we buy in a few years time will be genetically engineered strains that are (for instance) more resistant to attack by bacterial pathogens and that retain higher protein content during storage. This is not going to happen quickly, however, as transformation is not yet a routine procedure in any of these fungi and consumer acceptance of genetically modified foodstuffs is presently unenthusiastic, if not positively hostile. There will clearly be research in this direction nevertheless.

Secondly, molecular methods are revealing markers that allow the similarity/relatedness of strains to be defined with a resolution and certainty that has formerly been lacking (Loftus *et al.*, 1988). The cost of strain development can be sustained much more easily if the end-product can be commercially protected. Recent recognition of widespread wild *A. bisporus* and description of the genetic structure of these wild populations (see, e.g., Kerrigan *et al.*, 1998) has made conventional breeding again an attractive commercial proposition, particularly if the resultant strains can be commercially protected.

Thirdly, the growth and fruiting of these fungi is still a poorly understood area of microbiology that is being developed by molecular analyses far more rapidly than traditional genetic/physiological studies on their own could achieve. The better understanding of the way these organisms grow and develop is necessary knowledge for both improved cultivation methods and strain improvement.

2. GENES FROM THE CULTIVATED FUNGI

The genes listed in Tables 3–8 show the considerable range of aspects of fungal physiology which are currently being studied in the cultivated species. Sequence data have only so far been deposited for genes from *Agaricus bisporus*, *Pleurotus* species, *Lentinula edodes* and *Flammulina velutipes*. As Tables 3 and 4 show, most sequences have been obtained from *A. bisporus*. For the purposes of this article the sequences have been divided on the basis of whether they encode intracellular enzymes or secreted proteins.

2.1. Intracellular Enzymes

The genes from the edible basidiomycetes which code for intracellular proteins have largely been cloned with the aims of: (1) understanding processes within these fungi which may be of commercial importance, (2) identifying sequences

Table 3 Gene sequences: *A. bisporus* intracellular proteins.[a]

Gene	Function	Gene bank accession	Reference
gpd1	Glyceraldehyde-3-phosphate dehydrogenase	M81727	Harmsen *et al.* (1992)
gpd2	Glyceraldehyde-3-phosphate dehydrogenase	M81728	Harmsen *et al.* (1992)
	Lectin	U14936	Crenshaw *et al.* (1995)
eflA	Elongation factor 1-alpha	X97204	Sonnenburg *et al.* (1996)
hhfA	Histone H4	X94189	Sonnenburg *et al.* (1996)
htbA	Histone H2B	X94188	Sonnenburg *et al.* (1996)
rpaB	DNA-directed RNA polymerase 1	X94765	Sonnenburg *et al.* (1996)
	4-amino benzoate hydroxylase	D38415	Tsuji *et al.* (1996)
gdhA	NADP-dependent glutamate dehydrogenase	X83393	Schaap *et al.* (1996)
chsA	Chitin synthase	X98488	
sepA	Septin	Z82019	De Groot *et al.* (1997)
cypA	Cytochrome p450	Z82021	De Groot *et al.* (1997)
atpD	ATP-synthase delta subunit	Z82020	De Groot *et al.* (1997)
glnA	Glutamine synthetase	Y12704	Kersten *et al.* (1997)
hem1	Aminolaevulinic acid synthetase	Z50096	Yagüe *et al.* (1997)
pruA	1-pyrroline-5-carboxylate dehydrogenase	X95584	Schaap *et al.* (1997)
aac1	Adenylate cyclase	AF055295	Binz *et al.* (1998)
mtDH	NADP-dependent mannitol dehydrogenase	AF053764	Stoop and Mooibroek (1998)
sudA	Succinate dehydrogenase (Fe-S) subunit	Y15942	De Groot *et al.* (1999)
hspA	Heat shock protein (HSP70)	X98508	
palA	Phenylalanine ammonia lyase	Z82021	
echA	Peroxisomal enoyl-CoA hydratase	Y17825	
pkiA	Pyruvate kinase	X97579	
pgkA	3-phosphoglycerate kinase	X97580	
aldA	Aldehyde dehydrogenase	Y17825	
spr1	Serine proteinase	Y13805	
ppo1	Polyphenol oxidase	X85113	
ppo2	Tyrosinase	AJ223816	
abr1	Transposon-like element	Y18555	

[a]Some partial sequences usually obtained for taxonomic analysis or karyotyping are not included in this table, but are shown in Table 9. No reference indicates that there is an entry in the gene bank for the sequence but as yet there is no corresponding publication.

of use for phylogenetic purposes (which is not within the scope of this article), or (3) identifying markers with the intention of generating a physical linkage map of the genomes of these organisms.

Despite the achievements of the last decade, it is clear that little is known of the molecular genetics of edible basidiomycete metabolism. Although it is likely that much of what is known of fungal metabolism in general may be applied to the edible basidiomycetes, if rational breeding of improved strains is to become a reality (whether by conventional or molecular methods) much more information is required about the genes and enzymes involved in at least

Table 4 Gene sequences: *A. bisporus* secreted proteins.

Gene	Function	Gene bank accession	Reference
cel1	Cellulose growth-specific protein	M86356	Raguz *et al.* (1992)
cel2	Cellobiohydrolase	X50094	Yagüe *et al.* (1997)
cel3	Cellobiohydrolase	L24520	Chow *et al.* (1994)
cel4	Mannanase	Z50094	Yagüe *et al.* (1997)
abexg1	Exo-1,3-β-glucanase	X92961	van de Rhee *et al.* (1996b)
abexg2	Exo-1,3-β-glucanase	X92961	van de Rhee *et al.* (1996b)
xlnA	Endoxylanase	Z83199	De Groot *et al.* (1998b)
lcc1	Laccase	L10664	Smith *et al.* (1998)
lcc2	Laccase	L10663	Perry *et al.* (1993b)
hypA	Hydrophobin (HYPA)	X89242	De Groot *et al.* (1996)[a]
hypB	Hydrophobin (HYPB)	X15941	De Groot *et al.* (1996)[a]
hypC	Hydrophobin (HYPC)	X90818	De Groot *et al.* (1996)[a]
abh1	Hydrophobin (ABH1)	X92861	Lugones *et al.* (1996)[a]
abh2	Hydrophobin (ABH2)	X92860	Lugones *et al.* (1996)[a]
abh3	Hydrophobin (ABH3)	Y14602	Lugones *et al.* (1998)[a]

[a]The genes coding for the hydrophobins have been cloned independently by separate groups; *hyp A* corresponds to *abh1*, *hyp B* corresponds to *abh3* and *hyp C* corresponds to *abh2*.

Table 5 Gene sequences: *Pleurotus* intracellular proteins.

Strain	Gene	Function	Gene bank accession	Reference[a]
P. ostreatus		Ornithine carbamyltransferase	E10098	
P. ostreatus	*caM*	Calmodulin	U91643	
P. eryngii	*aao*	Aryl alcohol oxidase	AF064069	
P. ostreatus	*sdil-b*	Succinate dehydrogenase (Fe–S) subunit	AB007363	Irie *et al.* (1998)
P. djamor	*pcat1*	Catalase	U75450	
P. ostreatus	*pcat2*	Catalase	U75451	
P. sjor-caju		Beta-tubulin	AF132911	

[a]No reference indicates that there is an entry in the gene bank for the sequence but as yet there is no corresponding publication.

several key metabolic pathways. Also, there is now an increasing amount of evidence to suggest that mushrooms and/or mushroom products may have therapeutic value for treating diseases such as cancer, and this makes an understanding of their secondary metabolism essential (see, e.g., Parslew *et al.*, 1999). The development of fruit bodies and nitrogen metabolism are two processes that have been studied, and this progress is detailed below.

Table 6 Gene sequences: *Pleurotus* secreted proteins.

Strain	Gene	Function	Gene bank accession	Reference[a]
P. ostreatus	*pox1*	Diphenol oxidase, laccase	Z34847	Giardina *et al.* (1995)
P. ostreatus	*poxA1B*	Laccase	AJ005017	
P. ostreatus	*pox2*	Diphenol oxidase, laccase	Z49075	Giardina *et al.* (1996)
P. ostreatus	*pox3*	Laccase	U75452	
P. eryngii	*mnp1*	Manganese peroxidase	AF007221	Ruiz-Duenas *et al.* (1999)
P. eryngii	*mnp2*	Manganese peroxidase	AF007222	Martinez *et al.* (1996)
P. ostreatus	*mnp3*	Manganese peroxidase	AF011546	Asada *et al.* (1995)
P. ostreatus	*poh1*	Hydrophobin (POH1)	Y14656	Asgeirsdottir *et al.* (1998)
P. ostreatus	*poh2*	Hydrophobin (POH2)	Y14657	Asgeirsdottir *et al.* (1998)
P. ostreatus	*poh3*	Hydrophobin (POH3)	Y16881	Asgeirsdottir *et al.* (1998)
P. ostreatus	*fbh1*	Fruit-body hydrophobin 1	AJ004883	Penas *et al.* (1998)

[a]No reference indicates that there is an entry in the gene bank for the sequence but as yet there is no corresponding publication.

Table 7 Gene sequences: *Lentinula edodes* intracellular proteins.

Gene	Function	Gene bank accession	Reference[a]
priA	Developmentally regulated novel gene	X60956	Kajiwara *et al.* (1992)
mfbAc	Cell adhesion protein	D14487	Kondoh *et al.* (1995)
mfbBc	Fruit-body-specific novel gene	D14488	Ishibashi *et al.* (1996)
uck1	UMP-CMP kinase	AB005742	Kaneko *et al.* (1998)
cap	Adenyl cyclase-associated protein	AB001578	Zhou *et al.* (1998)
shp1	Small heme-binding protein	AB015310	

[a]No reference indicates that there is an entry in the gene bank for the sequence but as yet there is no corresponding publication.

Table 8 Gene sequences: *Flammulina velutipes* intracellular proteins.

Gene	Function	Gene bank accession	Reference[a]
fds	Fruit-development-specific gene	D83744	Azuma *et al.* (1996)
fvfd16	Fruit-development-specific gene	D88441	

[a]No reference indicates that there is an entry in the gene bank for the sequence but as yet there is no corresponding publication.

2.1.1. Genes Involved in Fruiting Body Development

At first sight, most of the genes in Table 3 could be classified as housekeeping genes. Genes for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, *gpd,* and aminolaevulinic acid synthetase, *hem1* (haem biosynthesis), have both been employed as constantly expressed controls in expression studies (Schaap *et al.*,1997; Yagüe *et al.*, 1997) and so far appear to be good choices for this purpose. For other genes listed, however, expression is often closely linked with developmental events in the fungus. The very rapid biomass production/transfer which occurs during fruiting appears to require the differential expression of several, and probably many, genes and attempts are under way to identify these sequences. Early observations that enzymes involved in carbon metabolism such as glucose-6-phosphate dehydrogenase and mannitol dehydrogenase have optimal enzyme activity at the onset of fruiting generated much interest in these enzymes (Hammond and Nichols, 1976; Hammond, 1981). The gene encoding mannitol dehydrogenase from *A. bisporus* has been cloned and sequenced and its expression would appear to be related to fruit body development, but there is also a response to salt stress, indicating a general function in osmoregulation (Stoop and Mooibroek, 1998). Glucose-6-phosphate dehydrogenase has also recently been cloned but as yet no expression data are available (P.J. Schaap *et al.*, unpublished data). Differential display methodology has been applied to *A. bisporus* with the aim of determining which genes are involved in fruiting (De Groot *et al.*, 1997). In this study three genes, *atpD*, *cypA* and *sepA,* encoding an ATP synthase subunit, a cytochrome p450 and septin, respectively, were all found to be up-regulated during fruiting. Two other upregulated genes were identified but their function is unknown. Five other genes were found to be fruit body-specific in this study, but the only identifiable sequence was *hypA* which encodes a hydrophobin (De Groot *et al.*, 1997; see section 2.2.2). Genes involved in fruiting have also been cloned in *Lentinula edodes* but the functions of these sequences have yet to be elucidated (Azuma *et al.*, 1996). The post-harvest physiology, and in particular senescence and browning of fruit bodies, are also of commercial importance. Studies on a fruit-body-specific serine proteinase from *A. bisporus* (Burton *et al.*, 1993; Burton *et al.*, 1997a) and a number of tyrosinase isoforms (van Leuwen and Wichers, 1999) have shown that these proteins are involved in post-harvest changes. Although the *spr1* encoding the serine proteinase and the *ppo1* and *ppo2* (tyrosinase) genes have sequences deposited in the databases, there are few published results relating the genetics to the expression and physiological properties of their gene products.

2.1.2. Nitrogen Metabolism

A. bisporus can utilize ammonium as a sole nitrogen source, and both glutamine synthetase and an NADP-dependent glutamate dehydrogenase have been characterized (Baars *et al.*, 1994; Baars *et al.*, 1995a, b). The genes for these enzymes have also been sequenced: *glnA* encodes glutamine synthetase (Kersten *et al.*, 1997) and *gdhA* encodes the NADP-dependent glutamate dehydrogenase (Schaap *et al.*, 1996). Less is known about organic nitrogen metabolism, although genes encoding a cytosolic pyrroline-5-carboxylate dehydrogenase (*pruA*: Schaap *et al.*, 1997) and δ-amino-laevulinic acid synthetase (*hem1*: Yagüe *et al.*, 1996) have been cloned.

2.2. Secreted Proteins

2.2.1. Substrate Utilization Proteins

One of the defining properties of the filamentous fungi is their ability to secrete a broad range of proteins into their surrounding environment (Berbee and Taylor, 1999).

All of the edible basidiomycetes are grown on lignocellulose-containing materials, and it is the enzymes involved in utilization of these substrates that have been examined extensively (but almost exclusively in other fungi; see, e.g., reviews by Coughlan *et al.*, 1993; Gold and Alic, 1993; Bhat and Bhat, 1997; Cullen, 1997; Smith *et al.*, 1997). With edible species, most progress in this area has been made on *A. bisporus* (Table 4), although work on enzymes of substrate utilization by some *Pleurotus* species is accumulating (Table 6).

Mushroom compost consists of two main components, lignocellulosic residues and microbial biomass (Wood and Smith, 1987). *A. bisporus* has been shown to produce a range of enzymes capable of degrading these components, including endoglucanases, cellobiohydrolases, β-glucosidase, xylanases, mannanases, laccase (Wood, 1989) and Mn-dependent peroxidases (Bonnen *et al.*, 1994) which are all involved in the degradation of plant polymers. Microbial biomass-degrading enzymes so far observed include nucleases, proteinases, lipases, muramidase, chitinase, β-glucanase and *N*-acetyl-β-glucosaminidase (Puls *et al.*, 1987; Fermor *et al.*, 1991; Wood and Thurston, 1991; Yagüe *et al.*, 1996). Some of these enzymes may be involved in several processes, including degradation of material from both plant and microbial sources and autolysis.

Studies on lignin degradation by *A. bisporus* have focused mainly on the enzyme laccase. Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.3) is produced in great quantities by the organism. It constitutes some 2% of

mycelial protein during vegetative growth (Wood, 1980). The enzyme is a typical fungal laccase with a subunit molecular mass of 65 kDa and is unusual only in that it functions as a dimer (Perry *et al.*, 1993a; Leontievsky *et al.*, 1997). Two laccase genes, *lcc1* and *lcc2*, have been cloned and sequenced (Perry *et al.*, 1993b). *lcc1* lies 1.3 kb downstream of *lcc2*, and sequence analysis has shown that *lcc2* corresponds to the peptide sequence of the (major component of) pure protein (Perry *et al.*, 1993b; Smith *et al.*, 1998). Since the two genes were isolated from a cDNA library it is clear that *lcc1* is also expressed at least at the mRNA level. *lcc1* message has been quantified by competitive RT-PCR and this has shown that, although *lcc1* message is produced in mycelium colonizing compost, its level is vastly (*ca.* 7000 times) less than that of *lcc2* mRNA (Smith *et al.*, 1998). The function of *lcc1* remains unclear, but there are now a number of fungal systems where extracellular enzymes are encoded by gene families of two to many, often very similar, genes that show striking (yet unexplained) differences in level of expression of what are apparently very similar proteins (e.g. Bogan *et al.*, 1996a,b; Gettemy *et al.*, 1998).

Laccases have also been characterized in both *L. edodes* and *Pleurotus* strains. Two laccases have been purified from *Pleurotus eryngii* with molecular masses of 65 and 61 kDA, respectively (Munoz *et al.*, 1997). Work on *Pleurotus ostreatus* has shown the presence of at least two laccase genes, *pox1* and *pox2*. As with the *A. bisporus lcc1* and *lcc2* genes, *pox1* and *pox2* are not allelic and appear to be part of a multigene family (Giardina *et al.*, 1995). Comparison with sequence of purified laccase protein from *P. ostreatus* revealed that *pox2* encodes a previously purified and characterized laccase (Palmieri *et al.*, 1993; Giardina *et al.*, 1996). Laccase has also been purified from *L. edodes* and fragments of laccase genes have been isolated and sequenced (Leatham and Stahmann, 1981; Kofujita *et al.*, 1991; D'Souza *et al.*, 1996). To date no complete laccase gene sequences from *L. edodes* are present in the databases.

Agaricus bisporus produces a manganese-dependent peroxidase which is also thought to be involved in lignin degradation. The enzyme has been purified and N-terminal sequencing and antibody production have been performed with the purified protein (Bonnen *et al.*, 1994; P. Lankinen, personal communication) but no related gene sequence is available. Studies on *P. ostreatus* and *P. eryngii* manganese peroxidases have been more fruitful. To date one manganese peroxidase gene has been found in *P. ostreatus* and two others have been found in *P. eryngii* (Asada *et al.*, 1995; Ruiz-Duenas *et al.*, 1999). These gene sequences share homology with other fungal manganese peroxidases, but detailed information on the regulation and exact biochemical function of these genes is absent.

A. bisporus produces a complete cellulase system which enables it to grow on crystalline cellulose as the sole carbon source (Manning and Wood, 1983).

So far four cellulose-growth-dependent genes have been isolated, *cel1*, *cel2*, *cel3* and *cel4* (Raguz *et al.*, 1992; Chow *et al.*, 1994; Yagüe *et al.*, 1996). The *cel1* gene product possesses a cellulose binding domain, a serine, threonine, proline-rich linker region and a catalytic core sequence showing homology to endoglucanase 1 and cellobiohydrolase 2 of *Trichoderma reesei* (Raguz *et al.*, 1992). The core sequence of the gene has recently been shown to be homologous to *T. reesei egIV* (endoglucanase 4), and these two genes have been placed into a new family of the glycan hydrolases, family 61 (Saloheimo *et al.*, 1997). *cel2* and *cel3* are homologous to *cbh1* and *cbh2* of *T. reesei* and belong to families 7 and 6 of the glycosyl hydrolases, respectively (Chow *et al.*, 1994; Yagüe *et al.*, 1996). *cel4* is homologous to a β-mannanase from *T. reesei* and has been cloned and expressed in both *Pichia pastoris* and *Saccharomyces cerviseiae*. Enzymological studies of these recombinant yeasts have shown that *cel4* does indeed encode a protein with β-mannanase activity (Tang *et al.*, 1998). Thus present information suggests that the cellulolytic enzyme machinery of *A. bisporus* is broadly similar to that of the well-studied cellulolytic ascomycetes (Penttilä and Saloheimo, 1999).

The cellulase system of *A. bisporus* is induced or repressed depending on the carbon source. Easily metabolized carbon sources such as glucose repress cellulase production, whereas solka floc and crystalline cellulose induce cellulase production. Northern analysis on all four genes has shown that *cel* mRNAs are produced during growth on cellulose but their synthesis is rapidly repressed upon addition of glucose (Yagüe *et al.*, 1997). Nuclear run-on analysis has shown that the rate of transcription of *cel1* and *cel3* in cellulose-grown cultures is 6 and 13 times that of cultures grown in glucose, respectively (Chow *et al.*, 1994; Yagüe *et al.*, 1994). The half-life of *cel* mRNA is apparently very short when the inducer is removed and/or the repressor added; for example *cel1* mRNA disappeared in less than five minutes after the removal of the inducer (Yagüe *et al.*, 1997). Two possible conclusions can be drawn from this: either *cel* mRNA is stabilized under inducing conditions, or the RNA simply has a high turnover rate (Yagüe *et al.*, 1994, 1997). In *A. bisporus* grown on compost, cellulase activity in the compost and *cel3* mRNA in compost mycelium are maximal during fruit enlargement, and this is thought to be associated with a need for carbon and energy to produce the (very rapidly enlarging) fruit body (Claydon *et al.*, 1987; Ohga *et al.*, 1999). Much less is understood of the cellulolytic capacity of the other edible basidiomycetes. Physiological studies on *P. ostreatus*, however, suggest a similar system of regulation to that of *A. bisporus* as complex carbon sources induce cellulase activity (Garzillo *et al.*, 1994).

The third constituent of lignocellulose is the complex and variable mixture of heterogeneous sugar polymers traditionally called hemicellulose (Tinnel, 1967). Apart from the work on mannanase (CEL4) described above, studies with *A. bisporus* have focused on the enzymes involved in the degradation of

xylan. *A. bisporus* produces at least three endoxylanase activities, and as with the cellulases these appear to be induced by complex substrates (both cellulose and xylan induce) and repressed by more easily metabolized carbon sources such as glucose (Whiteford *et al.*, 2000). An endoxylanase gene, *xlnA*, has been cloned that is apparently most strongly induced during growth in compost culture (De Groot *et al.*, 1998b). The production of endoxylanases, like cellulases, appears to be linked to developmental stages of *A. bisporus* during growth and development on compost. This would suggest a partial co-regulation of xylanases and cellulases, but these regulatory circuits are far from being completely understood (Whiteford *et al.*, 2000).

In comparison to lignocellulolysis, much less is understood about the process by which the edible basidiomycetes degrade the microbial biomass component of their growth substrate. Only some extracellular proteinases (Burton *et al.*, 1997b), muramidase and *N*-acetyl glucosaminidase (Lincoln *et al.*, 1997) from *A. bisporus* have been studied in any detail and as yet there are no genetic data relating to these enzymes. Many of these enzymes may well have an additional function in autolysis. Understanding of this process will be enhanced by the cloning of two exo-1,3-β glucanase genes (van de Rhee *et al.*, 1996b) and the purification of an endo-1,3-β-glucanase (Galan *et al.*, 1999) from *A. bisporus*, which are likely to be autolytic.

Considering the volume of data relating to substrate utilization by *A. bisporus*, there are considerable gaps in our understanding of the molecular genetics of this process. The multiplicity of genes involved presents problems, not only in elucidating the exact biochemical function of a given lignocellulolytic gene, but also in understanding how they all interact and the factors governing their regulation.

2.2.2. Hydrophobins

A second group of secreted proteins found in the edible basidiomycetes attracting much attention in the past few years are the hydrophobins. Hydrophobins are small secreted proteins which polymerize to form amphipathic films when in contact with hydrophilic–hydrophobic interfaces (Wessels, 1994) and are present as assemblages on emergent hyphal structures (Wessels, 1997). Although homology between amino acid sequences of different hydrophobins can be low, they are characterized as having a conserved spacing of eight cysteine residues.

Hydrophobins can be categorized into two classes on the basis of their solubility in sodium dedecyl sulphate (SDS). Class I hydrophobins such as SC3 from *Schizophyllum commune* (Schuren and Wessels, 1990) and ABHI from *Agaricus bisporus* (Lugones *et al.*, 1996) form stable insoluble complexes in the presence of SDS, whereas class II hydrophobins form unstable SDS soluble

films (Richards, 1993) – an example being, cerato-ulmin from *Ophiostoma ulmi* (Bowden *et al.*, 1993). There is considerable biotechnological interest in the use of hydrophobins as coatings on hydrophobic surfaces as an intermediate for attachment of cells or antibodies, and as coatings to vesicles for drug delivery systems (Wessels, 1997). The very interesting physical characteristics and functions of fungal hydrophobins have been reviewed by Wessels (1997).

Three hydrophobin genes have been identified in both *A. bisporus* and *P. ostreatus*. *abh1* (*hyp A*) encodes a hydrophobin which is expressed in fruit body tissue in *A. bisporus* (De Groot *et al.*, 1996; Lugones *et al.*, 1996). The protein consists of 112 amino acid residues and has a monomer molecular mass of around 9 kDa. Most *abh1* (*hyp A*) mRNA and protein is found on the outer peel tissue of the fruit body, although immunolocalization experiments have shown ABH1 to be present throughout the fruit body, with only gill tissue being completely unreactive to anti-ABH1 antibodies (Lugones *et al.*, 1996). The protein is a class I hydrophobin and assembles into characteristic rodlet structures both *in vitro* and *in vivo* (De Groot *et al.*, 1996; Lugones *et al.*, 1996). The data would suggest that ABH1 is solely responsible for the hydrophobicity of both internal and external surfaces within mushrooms that are in contact with the air (Lugones *et al.*, 1996, 1999). This hydrophobic interface prevents inflow of water into the fruit body and provides protection against fungal and bacterial attack (Lugones *et al.*, 1996).

The related sequence *abh2* (*hyp C*) is located 2.5 kb downstream of *abh1* and is also expressed in fruiting tissue but at much lower levels than *abh1* (*hyp A*) (De Groot *et al.*, 1996; Lugones *et al.*, 1996). The function of this hydrophobin is thought to be similar to that of ABH1 (De Groot *et al.*, 1996). In contrast, the hydrophobin encoded by *abh3* (*hyp B*) is expressed in aerial hyphae and mycelial chords (typically formed abundantly in the 'casing' layer applied to colonized compost to provoke fruit body formation).

No ABH3 has been found in association with fruiting tissue, although the differential expression of ABH3 may be under the control of mating type genes. ABH3 has a molecular mass of 19 kDa and is also a class I hydrophobin. The fact that it coats mycelial chords within the substrate suggests that ABH3 may also have a role in lignocellulolysis, since monomeric ABH3 could render lignin more hydrophilic, thereby promoting a much closer association between hyphae and substrate. The fact that class I hydrophobin membranes possess pores through which lignocellulolytic enzymes could pass supports this (Lugones *et al.*, 1998).

The hydrophobin genes cloned to date from *P. ostreatus* all have class I characteristics (Table 6). *POH1* is expressed only in fruiting bodies, whereas *POH2* and *POH3* are expressed in vegetative mycelium. A fruiting-body-specific hydrophobin, FBH1, has been purified and its N-terminal sequence determined; interestingly although the sequence is similar, it does not correspond exactly to that encoded by *POH1* (Penas *et al.*, 1998). POH2 is unusual

in that it is glycosylated; SC3 from *Schizophyllum commune* is the only other glycosylated hydrophobin found to date. POH2 and POH3 appear to perform a similar function to that of ABH3, by together forming a hydrophobic rodlet layer on aerial hyphae. POH1 probably fulfils the same role on the *P. ostreatus* fruiting body as ABH1 on the *A. bisporus* fruiting body (Asgeirsdottir *et al.*, 1998).

Phylogenetic analysis of the available basidiomycete hydrophobin sequences has shown that the relatedness of these genes corresponds to their functionality. Fruit-body-specific genes from different basidiomycetes are more closely related to each other than to the mycelium-specific hydrophobins within the same strains, and vice versa (Asgeirsdottir *et al.*, 1998).

3. STRAIN IMPROVEMENT OF CULTIVATED FUNGI

The unusual nature of the *A. bisporus* life cycle has meant that strain improvement by classical genetic means is very difficult. The main problem has been isolating sufficient quantities of vigorous, uninucleate, haploid propagules from the organism. Although haploid spores can be isolated by micro manipulation techniques (Elliot, 1972), the procedure is too time-consuming and unreliable for routine commercial use (Jin *et al.*, 1992). (Indeed, none of the cultivated species is a facile organism for classical genetic analysis.) As a result, virtually all current production of *A. bisporus* is based on a very small number of hybrid strains of little genetic diversity (Loftus *et al.*, 1988; Kerrigan *et al.*, 1998).

It is conjectured that the use of recombinant DNA technology will enable significant strain improvement in this and the other cultivated mushrooms, but it is by no means the only contribution to strain improvement that molecular genetic studies have to offer.

The study of gene expression (of which the first faltering steps are described above) is essential for understanding the processes of substrate utilization and fruit body formation, whether they are manipulated genetically or physiologically.

Secondly, molecular studies offer unique precision of strain identification, enabling a new level of management of commercial strains to be contemplated. In the past it has not been possible to retain effective ownership of valuable production strains because their unique identity could not be established (Loftus *et al.*, 1988). At present, work is under way to produce a detailed genetic linkage map in which important commercial traits can be linked to well-defined genetic markers. The separation of *A. bisporus* chromosomes using CHEF electrophoresis is now a well-established process (Lodder *et al.*, 1993; Sonnenberg *et al.*, 1996). The *A. bisporus* genome is estimated to be

Table 9 Chromosome markers in *Agaricus bisporus*.

Gene	Gene product or function	Chromosome	Reference[a]
tefA	Translation elongation factor	I	Sonnenberg *et al.* (1996)
MAT	Mating type	I	Sonnenberg *et al.* (1996)
ubiA	Poly-ubiquitin, protein 3	I	
ubiB	Ubiquitin B	I	
pkiA	Pyruvate kinase	I	
BSN	Basidial spore number	I	Imbernon *et al.* (1996)
atpD	δ-ATP synthase subunit (mitochondrial)	II	Schaap *et al.* (1995)
sepA	Septin	II	De Groot *et al.* (1997)
grpA	Glycine-rich RNA binding protein	II	
rabA	GTP-binding protein	III	
cel3	Cellobiohydrolase	III	Chow *et al.* (1994)
pgkA	3-phosphoglycerate kinase	III	Schaap *et al.* (1997)
hypA	Hydrophobin A	III	De Groot *et al.* (1996)
htbA	Histone H2B-1	III	Sonnenberg *et al.* (1996)
hspA	Heat shock protein A	III/IV	
rpaB	RNA polymerase I subunit β	IV	Sonnenberg *et al.* (1996)
pruA	Pyrroline 5-carboxylate dehydrogenase	IV	Schaap *et al.* (1997)
htbA-2	Histone H2B-2	IV	Sonnenberg *et al.* (1996)
htbA-3	Histone H2B-3	IV	Sonnenberg *et al.* (1996)
mrl30	Mitochondrial ribosomal protein LP33	IV	
rpL3	Ribosomal protein L3	IV	
zwfA	Glucose-6-phosphate dehydrogenase	IV	
chsA	Chitin synthase	IV	
glnA	Glutamine synthetase	IV	Kersten *et al.* (1997)
xlnA	Endoxylanase	V	De Groot *et al.* (1998b)
sudA	Succinate dehydrogenase	VI	
hypB	Hydrophobin B	VI	De Groot *et al.* (1999)
hhfA	Histone H4-1	VI	Sonnenberg *et al.* (1996)
hhfB	Histone H4-2	VI	Sonnenberg *et al.* (1996)
gdhB	NAD^+-glutamate dehydrogenase	VII	Schaap *et al.* (1997)
htbB	Histone 2B-2	VIII	Sonnenberg *et al.* (1996)
rsi3A	Ribosomal protein RS13A	VIII	Sonnenberg *et al.* (1996)
s15a	Ribosomal protein S15a	IX	Schaap *et al.* (1995)
l41A	Ribosomal protein L41	IX	Sonnenberg *et al.* (1996)
stpA	Sugar transport protein	IX	
28s rDNA	Large-subunit rRNA	IX	Schaap *et al.* (1996)
gtiA	General transcription initiation factor	IX	
gdhA	$NADP^+$-glutamate dehydrogenase	X	Schaap *et al.* (1996)
cel 1	Endoglucanase	X	Raguz *et al.* (1992)
alcA	Alcohol dehydrogenase	X	
cel 2	Cellobiohydrolase	X	Yagüe *et al.* (1996)
lcc 1	Laccase	XI	Perry *et al.* (1993b)
cypA	Cytochrome P450	XI	De Groot *et al.* (1997)
aldA	Aldehyde dehydrogenase	XII	
gpd1	Glyceraldehyde 3-phosphate dehydrogenase	XIII	Harmsen *et al.* (1992)

[a]No reference indicates that there is not as yet a corresponding publication.

37 megabases of sequence distributed amongst 13 chromosomes (Lodder *et al.*, 1993). To date some 45 *A. bisporus* genes have been mapped to these chromosomes and these are summarized in Table 9. Similar work performed on *Pleurotus ostreatus* has shown its genome to consist of 11 chromosomes ranging from 1.4 to 4.7 Mbp. The complete genome is estimated to be around 35 Mbp, although previous estimates were slightly smaller (Peberdy *et al.*, 1993; Larraya *et al.*, 1999). Only *pox1* and *fbh1* of the sequences with known function from *Pleurotus* have been localized to chromosomes. *pox1* hybridized to chromosome VI and *fbh1* hybridized to chromosome XI (Larraya *et al.*, 1999). Obviously more of the genes cloned from *Pleurotus* must be localized to chromosomes before progress in generating a linkage map can be made.

Interestingly, in both *A. bisporus* and *Pleurotus*, the size of several of the chromosomes is variable from strain to strain, probably indicating quite volatile amplification of repeat sequences (Sonnenberg *et al.*, 1996; Larraya *et al.*, 1999).

One of the major problems hampering progress in study of the molecular genetics of the cultivated mushrooms has been the failure to devise a reliable method for transforming these fungi. Although systems have been developed for other basidiomycetes such as *Schizophyllum commune* and *Coprinus cinereus* (Munaz-Rivas *et al.*, 1986; Binnenger *et al.*, 1987), transformation of *A. bisporus*, *P. ostreatus* and *Volvariella volvacea* remains an achievement as opposed to a process. Electroporation of protoplasts has been used with some success on *A. bisporus*. The system has enabled both the generation of hygromycin-resistant transformants (van de Rhee *et al.*, 1996a) and the homologous integration of genomic DNA into the *A. bisporus* genome (van de Rhee *et al.*, 1996b). Hygromycin-resistant *A. bisporus* transformants were also generated using a system involving *Agrobacterium tumefaciens* (De Groot *et al.*, 1998a). However, both methods have yet to be duplicated or extended in other laboratories. *Pleurotus ostreatus* and *Volvariella volvacea* have also been transformed using a chemically based method involving protoplasts (Hua Jia *et al.*, 1998).

It is unclear why basidiomycetes in general, and specifically the cultivated fungi, appear so recalcitrant to transformation. Whatever the reasons, a reliable transformation system would greatly facilitate study of the genes from these organisms, aside from the commercial opportunities for incorporation of desirable traits into a significant cash crop.

4. GENOMIC CHARACTERISTICS

Analysis of the genomic sequences of the cultivated basidiomycetes shows that, in common with other fungi, open reading frames are punctuated

frequently by short (50–90 bp) introns. These introns mostly start with a conserved GT**G sequence (the asterisks indicate any base) and end with A,T or C followed by AG. The conservation of intron boundary motifs is probably less in basidiomycetes than in the ascomycetes so far studied. Analysis of coding regions from the edible basidiomycetes has shown no marked codon bias, although in *A. bisporus* a slight preference for C and T is apparent at the third position (Whiteford, 1998).

Despite the rapidly accumulating amount of sequence from these organisms, no large fragments (>10 kb) of genomic DNA have been sequenced. As a result relatively little is known about how these genes are organized on chromosomes and the nature of intergenic sequences. However, the regions lying between *lcc1* and *lcc2* (Smith *et al.*, 1998), *hyp A* and *hyp B* (De Groot *et al.*, 1996) and *abexg1* and *abexg2* (van de Rhee *et al.*, 1996b) in *A. bisporus* have been sequenced. The close proximity of these genes has enabled comparison not only of the coding sequence but also of the 5′ non-coding sequence. The 5′ non-coding regions of *lcc1* and *lcc2* show 61% identity (over about 450 bp upstream of the ATG start codon) and both sequences share a number of putative transcription-factor binding sites. Although *lcc1* possesses two TATA sequences, only one is found in the *lcc2* non-coding sequence (Smith *et al.*, 1998). From superficial comparison of the two promoter regions, there is no obvious reason why the two genes are expressed so differently. The 5′ non-coding regions of sequence of *hyp A* and *hyp C* also show considerable homology, and as with *lcc1* and *lcc2* share many common features (De Groot *et al.*, 1996).

5. CONCLUSION

Despite the identification of many putative transcription binding sites and other features of promoter regions in *A. bisporus*, *Pleurotus* and *Lentinula* species to date, there is no functional analysis of any of these promoter regions and the subsites within them. The use of transformation to enable knockout and anti-sense strategies for analysis of gene function and regulation is a major area for new research. Equally, the very large corpus of work on mating types, and the control of developmental processes by mating-type genes in inedible basidiomycetes (Horton and Raper, 1995; Casselton and Olesnicky, 1998), needs to be related to the cultivated species. The molecular genetics of cultivated mushrooms has at least made sufficient progress in its first decade to give some idea of the very large amount that remains to be done.

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The Intestinal Microflora: Potentially Fertile Ground for Microbial Physiologists

Gerald W. Tannock

Department of Microbiology, University of Otago, PO Box 56, Dunedin, New Zealand

ABSTRACT

The intestinal microflora provides opportunities for microbial physiological research. The metabolic interactions of bacterial inhabitants of the intestinal community, bacterial bioenergetics, preferential utilization of substrates as energy sources by specific bacterial species, and intercellular signalling are among the topics of challenging research awaiting the attention of microbial physiologists.

ABBREVIATIONS

BSH	Bile salt hydrolase
CFU	Colony-forming unit
PCR	Polymerase chain reaction
TCA	Taurocholate

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1. A PRIMER ON THE INTESTINAL MICROFLORA

The bodies of animals, including humans, are the homes of numerous microbial species (mostly bacteria) that form complex communities on the epidermis and in the interior of hollow organs that open to the exterior of the body by one or more orifices. These communities of microbes that inhabit the bodies of healthy animals are generally referred to, collectively, as the 'normal microflora'. The gastrointestinal tract harbours a particularly copious microbial community comprising hundreds of bacterial species, some of which are present in large numbers (10^{10} CFU per gram). The distribution of the microflora within gastrointestinal tracts varies according to animal species. The foregut (rumen-reticulum) of ruminants is colonized by a microflora containing obligately anaerobic bacteria, fungi and protozoa, whose metabolic activities are essential for the digestion of plant structural materials in the animal's diet. A profuse collection of bacteria is also present in the hindgut (caecum and colon) of ruminants, and in that of all other animal species that have been investigated. The contribution of these intestinal microbes to the well-being of the host is more speculative. Microbial colonization of the gastrointestinal tracts of mice, rats, pigs and fowl occurs from gastric to rectal regions, whereas in humans the microflora is confined to the distal parts of the intestinal tract: the ileum and colon (reviewed by Tannock, 1995).

Bacterial genera commonly detected in the colon of humans are listed in Table 1. It seems likely that not all of the intestinal inhabitants can be cultivated

Table 1 Examples of bacteria inhabiting the human colon.

Bacteroides: Gram-negative, non-spore-forming bacilli. Obligate anaerobes. Metabolic products include combinations of acetic, succinic, lactic, formic or propionic acids. If *N*-butyric acid is produced, *iso*-butyric and *iso*-valeric acids are also present.
Bifidobacterium: Gram-positive, non-spore-forming, non-motile bacilli, sometimes club-shaped or spatulate extremities. Obligate anaerobes. Acetic and lactic acids are produced primarily, in the molar ratio 3:2. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate 'shunt' metabolic pathway.
Clostridium: Gram-positive bacilli that form endospores. Obligate anaerobes.
Eubacterium: Gram-positive bacilli, non-spore-forming. Obligate anaerobes. Produce mixtures of organic acids including butyric, acetic and formic acids.
Fusobacterium: Gram-negative, non-spore-forming bacilli. Obligate anaerobes. *N*-butyric acid is produced but *iso*-butyric and *iso*-valeric acids are not.
Peptostreptococcus: Gram-positive cocci. Obligate anaerobes. Can metabolize peptone and amino acids.
Ruminococcus: Gram-positive cocci. Obligate anaerobes. Amino acids and peptides are not fermented. Fermentation of carbohydrates produces acetic, succinic and lactic acids, ethanol, carbon dioxide and hydrogen.
Lactobacillus: Gram-positive bacilli that produce lactic acid as the major fermentation product.



Figure 1 Scanning electron micrograph of human faeces. Note the numerous bacterial cells and the variety of bacterial morphotypes. About 50% of faecal mass consists of bacterial cells.

using traditional bacteriological methods, because the total microscopic count is about ten times higher than the CFU per gram (Langendijk *et al.*, 1995). Production of a complete catalogue of intestinal inhabitants therefore awaits the results of the application of molecular biological detection and identification methods (PCR amplification and sequencing of 16S rDNA; fluorescent DNA probes and *in situ* hybridization) to the microbial communities of the distal intestinal tract (O'Sullivan, 1999).

Despite an abundance of species, the intestinal microflora is a somewhat neglected area of microbiology. This may be because of the unattractive nature of the samples that must be examined (most human studies concern the microflora of faeces; Fig. 1) and that many of the bacterial inhabitants are extremely oxygen-sensitive, requiring the use of anaerobic gloveboxes for their culture and maintenance. The intestinal microflora is of considerable significance medically, however, and the reasons for this are summarized in Table 2.

The intestinal microflora, regardless of any medical or other applied implications, is worthy of scientific study. The microflora constitutes one of the most heterogeneous microbial communities occurring naturally, and the intestinal tract provides an ecosystem through which knowledge of microbial ecology can be advanced. There is much fundamental research to be carried out in relation to this ecosystem, and microbial physiologists could play a major role in these investigations. This chapter will review some areas of intestinal

Table 2 Medical significance of the intestinal microflora of humans.[a]

Problem	Details
A reservoir of infection	Urinary tract infections (mainly due to *Escherichia coli*)
	Post-surgical sepsis (mainly facultative anaerobes such as *E. coli* and obligate anaerobes such as *Bacteroides fragilis* in mixed infections)
	Acute purulent meningitis of infants caused by *E. coli*
A reservoir of antibiotic-resistant bacteria	A consequence of inappropriate prescribing of antibiotics in clinical medicine, and inappropriate use in animal husbandry
Perturbations of the microflora	Antibiotic-associated colitis (pseudomembranous colitis) due to proliferation of *Clostridium difficile*
	Contaminated small bowel syndrome; colonization of the small bowel by 'faecal bacteria'
Implicated in chronic inflammatory conditions	A probable aetiological role for the intestinal microflora in ulcerative colitis and Crohn's disease

[a]For further details, see Tannock (1999).

microflora research where input from microbial physiologists will be essential if knowledge in this area is to be advanced. All of these research areas relate to the intestinal tract of monogastric animals, principally humans and mice.

2. METABOLIC ACTIVITIES OF THE INTESTINAL MICROFLORA: SOUND AND FURY BUT SIGNIFYING NOTHING?

The colonic content (about 200 grams on average) of humans contains something like 6×10^{13} bacterial cells. The extent of bacterial metabolism required to maintain a community of this size must be considerable. Energy is generated by intestinal bacteria through fermentation of carbohydrates and amino acids under the prevailing anaerobic conditions of the intestinal contents. The metabolic products produced by the microflora as a whole are often used to monitor the overall functioning of the microbial component of the ecosystem (Table 3). Some of these chemicals are present in the animal intestinal tract only because of the presence of the microflora, so these are referred to as 'microflora-associated characteristics' (Midtvedt, 1985).

Table 3 Comparison of selected biochemical properties of the intestinal tracts of germfree and conventional animals.

Property	Conventional[a]	Germfree[b]
Bile acid metabolism	Deconjugation, dehydrogenation and dehydroxylation	Absence of deconjugation, dehydrogenation, and dehydroxylation
Bilirubin metabolism	Deconjugation and reduction	Little deconjugation; absence of reduction
Cholesterol	Reduction to coprostanol	Absence of coprostanol
β-Aspartylglycine	Absent	Present
Intestinal gases	Hydrogen, methane and carbon dioxide	Absence of hydrogen and methane; less carbon dioxide
Short-chain fatty acids	Large amounts, several acids	Small amounts of a few acids
Tryptic activity	Little activity	High activity
Urease	Present	Absent
β-Glucuronidase (pH 6.5)	Present	Absent
Extent of degradation of mucins	More	Less
Enzyme activities associated with duodenal enterocytes	Lower	Higher
Serum cholesterol concentration	Lower	Higher

[a]Conventional = raised in association with a normal microflora; [b]germfree = raised in the absence of demonstrable microbes. All other factors were equal in these comparative studies.

What is the consequence of the presence of bacterial products in the intestinal contents? The significance of short-chain fatty acids, amino acids, vitamins and lipids of microbial origin to ruminant nutrition is well known, but the production of these molecules in the foregut is quite a different situation compared with that existing in the hindgut (colon; large bowel; McBee, 1977). In the ruminant, bacterial products are produced in the rumen which is anterior to the region of the gut where absorption of nutrients occurs (the omasum and small bowel). Short-chain fatty acids are absorbed from the large bowel, although their contribution to the energy requirements of a well-fed human is probably insignificant. Many animal species have retained relatively large caeca which comprise up to 5% of their bodyweight. Retention of this organ and its associated microbes is therefore presumably worthwhile in these cases because added bodyweight is detrimental in terms of energy expenditure for a speedy escape from predators. Unless an animal indulges in coprophagy, other

Table 4 Potential toxicological implications associated with the intestinal microflora.[a]

Activation of compounds to toxicants, mutagens, or carcinogens (azo compounds, nitro compounds, plant glycosides, 2-amino-3-methyl-3*H*-imidazo (4,5-*f*) quinoline)
Synthesis of carcinogens and mutagens (*N*-nitroso compounds, fecapentaenes)
Synthesis of promoters (bile acids, ammonia, phenols, cresols, fecapentaenes)
Enterohepatic circulation and deconjugation (steroid hormones, drugs, carcinogens)
Detoxification/protection (methylmercury, phyto-oestrogens, flavonoids)

[a]For further information, see Rowland (1999).

bacterial products produced in the large bowel may not be salvaged efficiently, if at all. A frequently cited benefit of bacterial metabolism in the human colon is the production of butyric acid which apparently provides an energy source for enterocytes forming the intestinal epithelium (Roediger, 1980).

While the 'beneficial' effects of the intestinal microflora are the most widely quoted in textbooks and reviews, there is a detriment to hosting complex communities of bacteria. Not all of their metabolic activities are desirable: bacterial metabolic products can be toxic to animal tissues (Table 4). Fortunately, the liver detoxifies these substances if they are absorbed from the digestive tract. Some detoxified substances are excreted in the urine, but others enter the intestinal tract in bile. Generally, these detoxified molecules have been conjugated to another molecule (such as glucuronic acid) so that they are more hydrophilic and less likely to be absorbed from the tract. Not to be beaten, it seems, some members of the microflora produce enzymes that deconjugate the molecules. Hence potentially toxic molecules can have an increased half-life in the animal body because they are re-absorbed and pass once again to the liver via the portal vein (an enterohepatic circulation). This ability of the microflora to chemically transform molecules produced endogenously, or indeed if ingested with the diet (xenobiotics), could be of importance toxicologically (Drasar and Barrow, 1985).

β-Glucuronidase is an enzyme produced principally by members of the genera *Bacteroides* and *Clostridium*, and which has received attention from microbiologists and toxicologists because of its potential to promote the enterohepatic circulation of molecules and to re-activate toxic molecules. The formation of glucuronides in the liver is a relatively common detoxification mechanism. Deconjugation of glucuronides in the intestinal tract is catalysed by bacterial β-glucuronidases (Drasar and Hill, 1974).

Although they do not produce the enzyme themselves, a group of Gram-positive, lactic acid-producing bacteria, the lactobacilli, appear to be able to influence β-glucuronidase activity in the large bowel. A reduction in the total β-glucuronidase activity of human faeces was observed by Goldin *et al.* (1980)

Table 5 Comparisons of enzyme activities in caecal contents of lactobacillus-free and lactobacillus-colonised mice.

Murine host	Gender	Mean enzyme activity (SEM)	
		Azoreductase	β-Glucuronidase
LF[a]	Male	4.9 (0.6)[b]	4949.0 (782.2)[c]
	Female	4.8 (0.5)	2381.5 (274.0)
LF + lactobacilli	Male	3.6 (0.6)	2827.8 (551.5)
	Female	3.1 (0.5)	2474.2 (485.5)

[a]Lactobacillus-free mice; [b]μmol of substrate reduced per h per g of caecal contents; [c]μmol *p*-nitrophenol released per h per g of protein (caecal contents).
For further information, see McConnell and Tannock (1991, 1993).

in subjects who were consuming milk containing the cells of an intestinal species of *Lactobacillus*. While mechanistically unexplained, this effect of lactobacilli has also been made using mice as experimental subjects (McConnell and Tannock, 1993). The effective demonstration of the impact of lactobacilli on the biochemistry of the intestinal tract has been largely made possible through the derivation of mice that harbour a gastrointestinal tract microflora from which lactobacilli are absent. The gastrointestinal microflora of conventional mice includes large populations of lactobacilli that are present throughout the digestive tract of the animals (Tannock, 1997). Therefore they provide the ideal animal species with which to study the influence of lactobacilli on the properties of the host. The derivation of lactobacillus-free mice from conventional animals was accomplished by the administration of antibiotics, inoculation of the mice with selected bacteria, and the use of gnotobiotic methodologies (Tannock *et al.*, 1988). Maintained in isolators, the mice provide a model system by which the influences of lactobacilli inhabiting the gastrointestinal tract can be determined. Comparisons of the characteristics of lactobacillus-free mice with counterparts that had been intentionally colonized with *Lactobacillus* strains, but in which an otherwise-identical microflora was present, have demonstrated that lactobacilli have major influences on intestinal biochemistry. An effect on β-glucuronidase activity is one such influence. β-glucuronidase activities were 50% higher in the caecal contents of male lactobacillus-free mice compared with animals colonized by lactobacilli of murine origin (Table 5; McConnell and Tannock, 1993). This elevated level of activity was not seen in female mice, suggesting that the amount of glucuronide substrates entering the digestive tract was different between males and females, since there is currently a lack of evidence to suggest that their microfloras differed in composition.

Similarly, the presence of lactobacilli in the intestinal tract has been observed to lower the total azoreductase activity of faecal or caecal samples. Azoreductase activity was 31% higher in the caecal contents of lactobacillus-free mice compared with that of animals colonized by lactobacilli (Table 5; McConnell and Tannock, 1991). This enzyme activity is of interest toxicologically because it catalyses the reductive cleavage of azo bonds such as those found in some food dyes. Hydrolysis of these molecules has been shown to produce mitogenic, and hence potentially carcinogenic, substances (Rowland, 1999).

How does the presence of lactobacilli in the intestinal tract produce these radical changes in enzyme activities? β-Glucuronidases and azoreductases have been detected in cultures of obligately anaerobic members of the intestinal microflora, but not in those of lactobacilli. The composition of the intestinal microflora does not appear to undergo major changes when lactobacilli are added to the ecosystem. Admittedly, newer molecular methods of analysis need to be applied to verify this, but the identification of the mechanisms by which lactobacilli influence the metabolic activities of other intestinal species obviously needs attention from microbial physiologists.

What does it all mean in terms of biological significance? It is fine to demonstrate changes in enzyme activity that are statistically significant, but the real question is whether the reductions make any difference to the long-term well-being of the animal. This aspect of intestinal microflora research has never been tackled adequately, leaving us wondering if this is all sound and fury but signifying nothing?

3. THE BILE SALT HYDROLASE ENIGMA: SUICIDAL TENDENCIES IN LACTOBACILLI?

Comparison of the characteristics of lactobacillus-free and lactobacillus-colonized mice revealed that lactobacilli are responsible for most of the bile salt hydrolase activity in the intestinal tracts of mice (Tannock *et al.*, 1989). Bile salt hydrolases catalyse the hydrolysis of conjugated bile acids, which results in the production of a free amino acid (taurine or glycine) and an unconjugated bile acid molecule. Conjugated bile acids enter the small bowel in bile and are important in the emulsification, digestion and absorption of dietary lipid that occurs in the proximal small bowel. Unconjugated bile acids are much less efficient in these roles (Drasar and Barrow, 1985). The concentrations of unconjugated bile acids in the small bowels of mice colonized by lactobacilli are higher than those of animals that do not harbour a *Lactobacillus* population (Table 6; Tannock *et al.*, 1994). Therefore bile salt hydrolases produced by lactobacilli are active in catalysing the deconjugation of bile acids in the small bowel environment. Fortunately, the decrease in the concentration of conjugated

Table 6 Percentage of unconjugated bile acids in small-bowel contents in relation to colonization of the gastrointestinal tract of mice by lactobacilli

Mouse group (*n*)	Total bile acids (mean, SEM)[a]	Percentage unconjugated bile acids (mean, SEM)
Lactobacillus-free (29)	22.8 (1.2)	23.5 (3.4)
Lactobacillus-colonized (23)	22.5 (2.5)	67.9 (4.6)

[a]Millimoles per kg (wet weight) of small-bowel contents (combined duodenal and jejunal contents).
For further information, see Tannock *et al.* (1994).

Table 7 Bile salt hydrolase activities of cell extracts of lactobacilli of murine origin.

Strain	Bile salt hydrolase activity[a]
Lactobacillus sp. strain 100–14	775, 1054
L. delbrueckii 21	394, 466
L. delbrueckii 18	253, 271
L. fermentum 20	20, 27
Lactobacillus sp. strain 100–93	0, 0

[a]Nanomoles of cholic acid released per mg protein per 30 min. Results of two assays are given.
For further information, see Bateup *et al.* (1995).

Table 8 A_{600} of cultures of lactobacilli after 24 h anaerobic incubation at 37°C.

Strain	BSH activity[a]	A_{600}	
		MRS[b]	MRS + TCA[c]
100-93	None	1.76	1.78
20	24	1.79	1.86
100-14	916	1.74	0.26
18	430	1.70	0.28
21	262	1.77	0.25

[a]Mean bile salt hydrolase activity (nanomoles of cholic acid released per mg of protein per 30 min); [b]Lactobacilli MRS medium; [c]7 mM taurocholate.
For further information, see Tannock *et al.* (1997).

bile acids in the small bowel owing to the production of bile salt hydrolase by lactobacilli does not affect the growth rate of the animals, which might have been a consequence if conjugated bile salt concentrations had fallen to a level suboptimal for lipid digestion and absorption (Bateup *et al.*, 1995).

The advantage to the lactobacilli of producing bile salt hydrolase in relation to colonization of the intestinal tract is not readily apparent. The bile acid nucleus is not degraded by lactobacilli, and taurine is not incorporated into bacterial cell proteins. There is variation in the amount of bile salt hydrolase activity associated with different strains of lactobacilli (Table 7), yet all of the strains are able to colonize the gastrointestinal tract of mice equally well (Bateup *et al.*, 1995). All current evidence points to an intracellular location for bile salt hydrolases, so conjugated bile salts must be transported into the lactobacillus cell (genes encoding a transport mechanism for taurocholate have been identified in *Lactobacillus johnsonii*; Elkins and Savage, 1998), deconjugated and the free bile acid returned to the external milieu (the free bile acid can be detected in the culture supernatant). It has been reported that the growth, *in vitro*, of a single strain of *Lactobacillus* that did not produce detectable amounts of bile salt hydrolase was stimulated by the presence of taurocholate in the culture medium (Tannock *et al.*, 1997). This observation does not support the proposal that deconjugation of bile acids is a detoxification mechanism (De Smet *et al.*, 1995). Indeed, free bile acids, such as cholic acid, are more toxic to the lactobacilli than are the conjugated forms in that *Lactobacillus* populations cease replicating and soon lyse once the concentration of cholic acid rises above 1 mM (Table 8) suggesting, anthropomorphically, that lactobacilli have suicidal tendencies. Some strains of lactobacilli are, nevertheless, more tolerant to the presence of cholic acid in the culture medium than are others (Tannock *et al.*, 1997). Perhaps there are differences in the efficiency of cell membrane-associated transport mechanisms between strains that can be used to maintain intracellular levels of cholic acid at non-toxic concentrations? Why do the lactobacilli chemically modify bile acid molecules at all? Can they gain energy from the transport of these conjugated and unconjugated molecules across membranes? Microbial physiologists interested in bioenergetics could probably profit from a study of these phenomena.

4. PREFERENTIAL UTILIZATION OF GALACTO- AND FRUCTO-OLIGOSACCHARIDES BY BIFIDOBACTERIA: DIETARY REGULATORS OF THE INTESTINAL ECOSYSTEM?

Members of the genus *Bifidobacterium* comprise about 0.8% of the total microscopic count of human faeces (generally considered to represent the

microflora of the colon; Langendijk *et al.*, 1995). Bifidobacteria have received modest microbiological attention because they are numerous in the faeces of human infants suckled at the breast, and persist as members of the microflora throughout life (Mitsuoka, 1992).

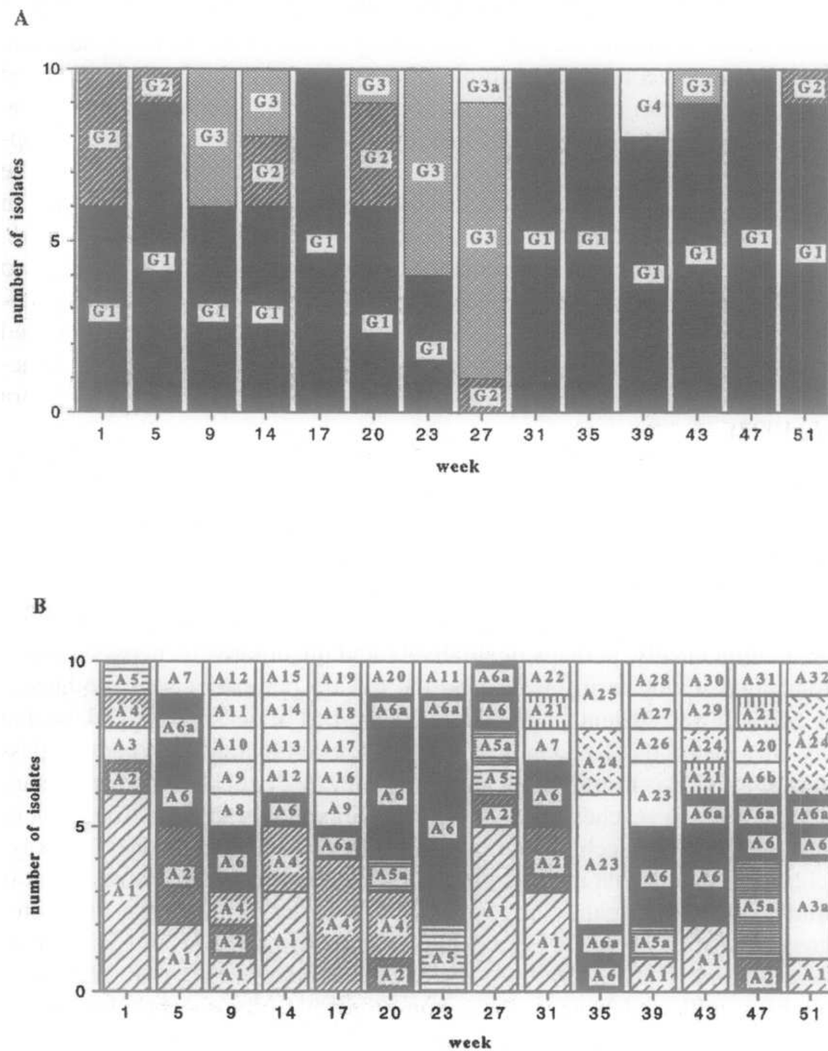


Figure 2 *Bifidobacterium* strains detected in faecal samples collected from two human subjects (A and B) over a 12-month period. Bifidobacterial strains from subject A were designated G1 to G4. Strains from subject B were designated A1 to A32. (Reproduced from McCartney *et al.* (1996) with permission.)

Genetic fingerprinting methods such as ribotyping and pulsed field gel electrophoresis of DNA digests prepared from bacterial isolates have permitted the analysis of the human faecal microflora at the level of strains. Examination of bifidobacterial populations in monthly faecal samples collected over a 12-month period from two healthy humans has shown that there can be marked variation in the complexity and stability of these bacterial populations between human subjects (McCartney *et al.*, 1996). In this study, one subject harboured a relatively simple (five strains of bifidobacteria detected during the 12-month period; Fig. 2A) and stable collection of bifidobacteria, but the other subject harboured 32 strains, some of which appeared, disappeared and sometimes reappeared during the course of the study (Fig. 2B). The collection of strains detected in each subject were unique to the individual, in that a strain common to both subjects was not detected (McCartney *et al.*, 1996). This study was later extended to a further ten healthy humans. Two faecal samples were obtained from each subject. About half of the subjects harboured a relatively simple bifidobacterial population and the others harboured a more complex collection of these bacteria. Unique collections of bifidobacteria that persisted throughout the study were detected in each subject (Kimura *et al.*, 1997).

What regulates the types and behaviour of bifidobacterial strains present in the microflora of a particular human being? Perhaps human genetic factors are involved, since differences in host physiology might directly or indirectly influence the types of bacteria colonizing the intestinal tract. For example, a large amount of substances that are potential nutrients for the intestinal bacteria are present in digestive tract secretions (Tannock, 1995). These may differ, at least antigenically, perhaps qualitatively and quantitatively, between hosts. Colonization might be influenced by the dietary components of the human host. Most of the nutrients in the diet are absorbed in the small bowel, so that really only materials that cannot be readily digested by human processes pass to the colon. This is mostly plant structural material, but about 10% of dietary protein apparently reaches the colon as well. Some foods derived from milk or plants contain oligosaccharides (galacto- or fructo-oligosaccharides) or polysaccharides (inulin) that are not digested in the small bowel. On average, it is estimated that Americans daily ingest 2.6 g of inulin and 2.5 g of fructo-oligosaccharides, about 70% of which comes from wheat and 25% from onions (Moshfegh *et al.*, 1999). As well as occurring naturally in foods (fruits, vegetables, milk and honey), these non-digestible oligosaccharides can be derived by enzyme-catalysed, industrial processes from various carbohydrates. They have become relatively common as additives in foods during the past decade, and are considered to confer health benefits on the consumer – in which case they are termed 'prebiotics' (Crittenden, 1999). Generally, oligosaccharides added to foods are not pure preparations, but contain a mixture of oligosaccharides of different degrees of polymerization, the parent

Table 9 Utilization of non-digestible oligosaccharides by bifidobacteria.

Bifidobacterial species	Oligosaccharides			
	Soybean	Raffinose	Stachyose	Fructose
B. bifidum	–[a]	–	+/–	–
B. longum	+++	++	+++	++
B. breve	+++	+++	+++	+
B. infantis	+++	+++	+++	++
B. adolescentis	++	++	++	++

[a]Relative amount of growth in presence of stipulated substrate.
For further information, see Mitsuoka (1996).

carbohydrate that was used as substrate, and monosaccharides. Non-hydrolysed oligosaccharides pass to the distal intestinal tract of humans where some members of the intestinal microflora are able to utilize them as fermentable energy sources.

Only a limited number of bacterial species that comprise the intestinal microflora have been screened for their ability to utilize the non-digestible oligosaccharides. Currently, it appears that bifidobacteria have the greatest capability to ferment the oligosaccharides (Crittenden, 1999). It would be of considerable interest to investigate non-digestible oligosaccharides, at the biochemical and physiological levels, in terms of their preferential utilization by different species or strains of bifidobacteria (Table 9). Would this explain the predominance of certain strains in the microflora of a particular human? Could the composition of the bifidobacterial population of an individual be modified by the administration of a specific oligosaccharide? What would be the consequence of this modification to the human host?

5. BACTERIOCINS: THE KILLING FIELDS OR INTERCELLULAR MESSENGERS?

The ability of bacterial cells to alert their kindred of increasing cell density (quorum sensing) is now well known. Established paradigms include intercellular signalling in the formation of fruiting bodies by myxobacteria (amino acids as signalling molecules), and the production of luminescence by *Vibrio fischeri* when colonizing the light organ of the squid, *Euprymna scolopes* (McFall-Ngai and Ruby, 1991; Kaiser and Losick, 1993; Greenberg, 1997). Indeed, the mechanism by which vibrio fluorescence is induced provides the

model for all signalling mechanisms involving *N*-acyl-homoserine lactones in the communication process. In this model, the *luxI* gene (or homologue) encodes the autoinducer (acyl-amino acid lactone) which is produced constitutively and diffuses out of the bacterial cell. As bacterial cell numbers increase, the extracellular concentration of the autoinducer increases until a critical level is reached. At this concentration, the autoinducer diffuses into the bacterial cells where interaction between the autoinducer and a transcriptional activator, encoded by the *luxR* gene (or homologue), leads in turn to expression of the genes encoding luminescence (or other activity). In the case of *V. fischeri*, it seems very likely that additional genes (other than those involved in luminescence) affecting colonization of the light organ are regulated by the same signalling process. Acyl-amino acid lactones are involved in signalling in many Gram-negative bacterial species, but Gram-positive bacteria utilize another approach.

Peptides figure largely as chemical signals among Gram-positive bacteria. Generally, the signalling peptide is secreted from the bacterial cell by a dedicated ATP-binding-cassette exporter. The secreted peptide functions as the input signal for a specific sensor component of a two-component signal-transduction system (Kleerebezem *et al.*, 1997). Nisin, produced by the dairy organism *Lactococcus lactis*, stimulates lactococcal cells to produce yet more of the substance. This system has been exploited rather elegantly to achieve heterologous gene expression in a strictly controlled manner: the promoter of the nisin structural gene is placed in front of the heterologous gene which is contained within an expression vector in a *L. lactis* strain. Addition of small amounts of nisin to the culture medium results in overproduction of the heterologous protein, whereas its production is negligible in the absence of nisin (Kuipers *et al.*, 1995). Peptides are involved in signalling between the cells of *Staphylococcus aureus*, leading to the expression of genes that encode proteins that may be important in pathogenesis, particularly in escaping from walled-off lesions (abscesses; Novick and Muir, 1999).

It is curious that many Gram-positive bacterial species produce peptides that, at an appropriate concentration *in vitro*, have antibacterial activities. These peptides have relatively narrow spectra of inhibitory activity and are referred to as 'bacteriocins' (Tagg *et al.*, 1976). It is generally assumed that bacteriocins are produced as antagonists and that they serve an ecological role by preventing potential competitors from co-colonizing a particular habitat. Nisin has antibacterial activity and, in a semi-purified form, is used as a 'preservative' in processed cheeses, in some countries.

The antibacterial activity of a known signalling molecule such as nisin raises the possibility that bacteriocins are produced by bacteria not with murderous intent, but as autoinducers that could be critical to colonization of particular habitats, including the gastrointestinal tract. Quorum sensing may be especially important to bacteria that form biofilms, as shown by the experiments of Davies and colleagues (1998). Normal biofilm formation by

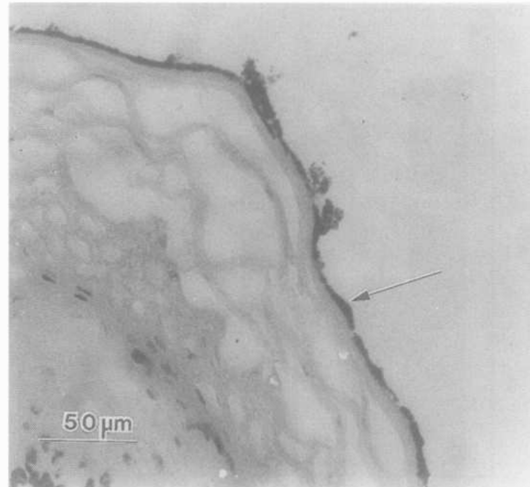


Figure 3 A Gram-stained section of the pars oesophagea of a porcine stomach. A biofilm composed of *Lactobacillus* cells is present on the epithelial surface (arrow). (Reproduced from Tannock (1992) with permission.)

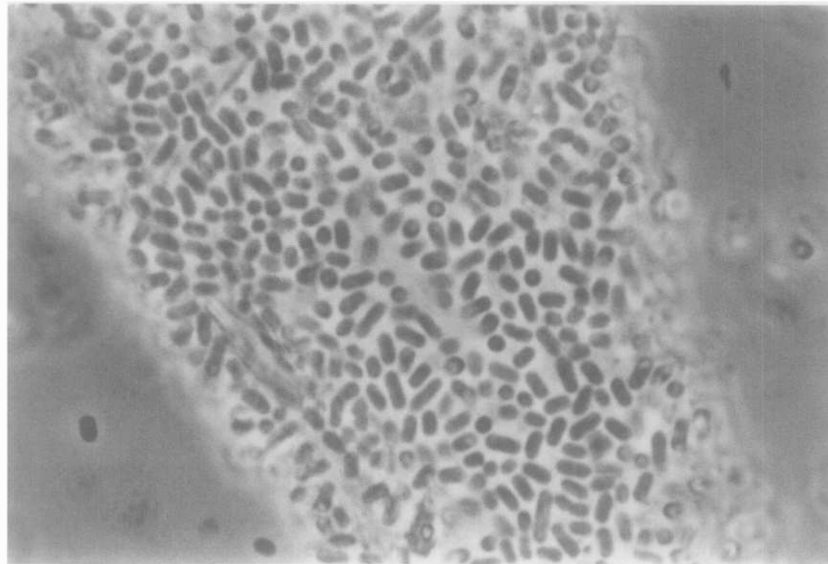


Figure 4 Cells of *Lactobacillus reuteri* strain 100–23 adhering to an epithelial cell harvested from the forestomach of a mouse. Phase-contrast microscopy.

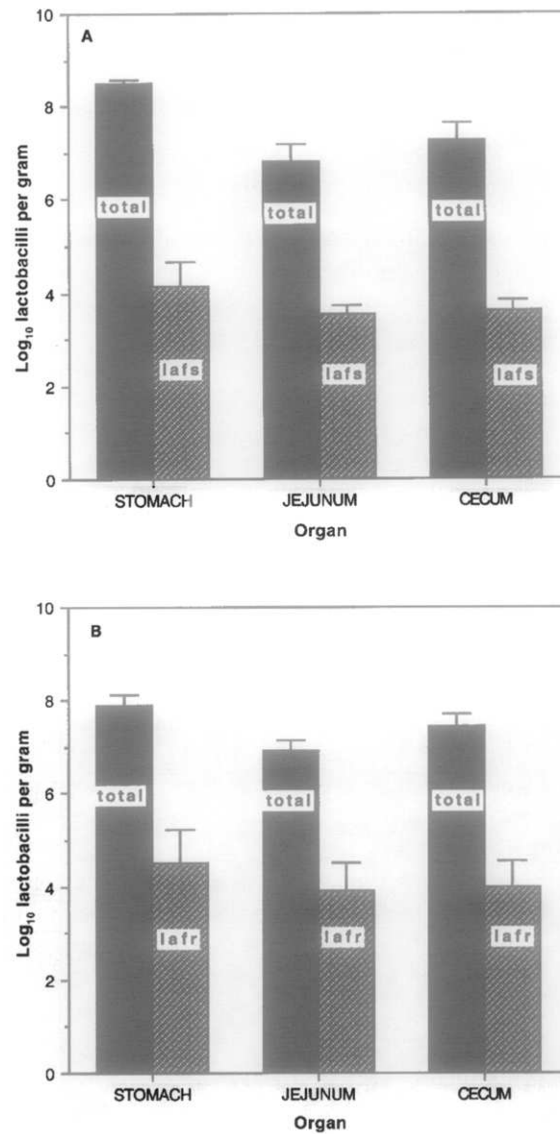


Figure 5 Results from two experiments in which lactobacillus-free mice were co-inoculated with isogenic strains of *Lactobacillus johnsonii*. A: mice inoculated with a lactacin F-producing, resistant strain and a non-producing, susceptible strain (lafs). B: mice inoculated with the lactacin F-producing, resistant strain and a non-producing but resistant strain (lafr). Total = total *Lactobacillus* population. The animals were examined two weeks after inoculation.

Pseudomonas aeruginosa on glass surfaces required the presence of an acyl-homoserine lactone signalling mechanism: mutant strains that could not signal produced thin, densely-packed films. Wild-type strains produced biofilms containing mushroom and pillar-shaped microcolonies separated by water-filled spaces. Addition of purified autoinducer to a biofilm produced by a mutant strain resulted in a return to normal biofilm structure.

Biofilms occur on certain epithelia lining proximal regions of the gastrointestinal tract of some animal species. These biofilms are composed of lactobacilli which adhere to stratified, squamous, epithelia of the mouse and rat forestomach, the porcine pars oesophagea, and the avian crop (Figs 3 and 4; Tannock, 1995). This kind of epithelium is non-secretory, so a blanket of mucus is not present on the surface of the epithelium and direct attachment of the lactobacilli to the epithelium, and to each other, is possible.

Lactacin F is a well-studied bacteriocin produced by *Lactobacillus johnsonii* VPI 11088 (Fremaux *et al.*, 1993). This strain of *Lactobacillus* forms a biofilm on the mouse forestomach epithelium. The bacteriocin is a 5.6 kDa, heat-stable, two-component (two peptides) substance with *in vitro* inhibitory activity against some other lactobacilli and *Enterococcus faecalis* (Allison *et al.*, 1994; Allison and Klaenhammer, 1996). In as yet unpublished experiments, the author and colleagues (D.M. Loach, G.E. Allison and T.R. Klaenhammer) have investigated the relative competitive abilities of isogenic strains of *L. johnsonii* in the gastrointestinal tract of lactobacillus-free mice. Briefly, lactobacillus-free mice maintained in isolators were inoculated with pairs of *Lactobacillus* strains: lactacin F-producing or non-producing; lactacin F-susceptible or resistant (based on *in vitro* observations). The populations of each strain could be selectively enumerated on culture media because they expressed different antibiotic-resistance genes. Thus the numerically dominant (most competitive) strain could be recognized in each colonization experiment. The lactacin F-producing, resistant strain predominated over a non-producing, susceptible strain, as might be expected if the bacteriocin was produced for antibacterial purposes (Fig. 5A). But the lactacin F-producing, resistant strain also predominated when tested with a non-producing, resistant strain (Fig. 5B), strongly suggesting that antibacterial activity was not the mechanism by which dominance was achieved by the producer strain. It is noteworthy that *luxI* and *luxR* mutants of *V. fischeri* fail to colonize the light organ of squids when wild-type vibrios are also present (M.J. McFall-Ngai and E.G. Ruby, personal communication). Therefore it seems that mutated signalling operons cannot be salvaged by the production of autoinducer by wild-type cells; the latter retain a competitive advantage. Could it be that lactacin F is a signalling molecule that aids in colonization of a gastrointestinal epithelium? Maybe the intestinal microflora will provide a rich source of information concerning communication within microbial communities?

6. MOLECULAR COMMUNICATION: BACTERIA CAN SPEAK TO ANIMALS

The intimate contact between the microbial community of the intestinal tract and the animal is a notable feature of this host–microbe relationship. Numerous microbial cells are confined within a relatively small, defined space and are separated from the sterile tissues of the host by an epithelium composed of a single layer of enterocytes. In the ileum of mice and some other animal species, filamentous segmented bacteria related to the clostridia (Snel *et al.*, 1994) attach by one end to enterocytes (Fig. 6), particularly in the vicinity of Peyer's Patches (Klaasen *et al.*, 1992). The microbes have not been cultivated, but ex-germfree mice monoassociated with them have been obtained (Klaasen *et al.*, 1991).

The method of attachment involves the insertion of the base of the filament into a murine enterocyte. Actin rearrangements within the enterocyte form a socket by which the filament becomes permanently attached to the mucosal surface (Jepson *et al.*, 1993). The mechanism by which the bacteria cause rearrangements of the enterocyte cytoskeleton are not known, but must involve the transmission of effector molecules from the bacterial cell to the enterocyte. This kind of 'communication' occurs between the intestinal pathogen, *Salmonella typhimurium*, and enterocytes. *Salmonella* cells in contact with the

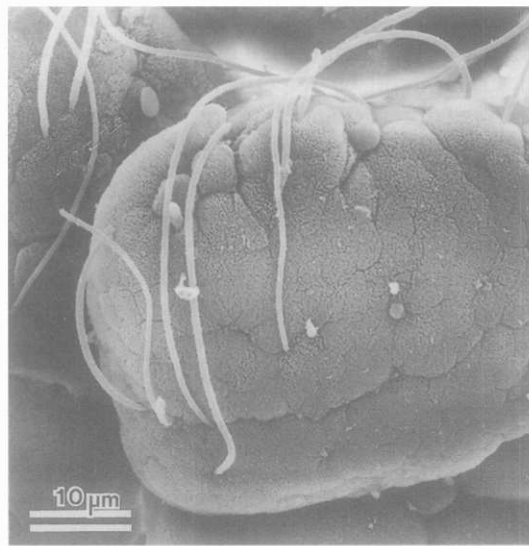


Figure 6 Scanning electron micrograph of a villus in the ileum of a mouse. Note the filmentous segmented bacteria attached by one end to the villous epithelium.

enterocyte surface express genes contained within a chromosomal 'pathogenicity island' that encode production of the type-III secretory pathway apparatus (contact secretion pathway). This forms a hollow flagellum-like structure that penetrates the enterocyte membrane (Kubori *et al.*, 1998). Virulence proteins (involved in the catalysis of actin depolymerization and repolymerization) pass directly from the bacterial cytosol to that of the enterocyte through the secretion apparatus. Cytoskeletal rearrangements lead to ruffling of the enterocyte membrane in the vicinity of the *Salmonella* cells and their ultimate internalization ('invasion'; Galan, 1996). The type-III secretory system has been detected in Gram-negative bacteria, but the filamentous segmented cells have Gram-positive bacterial wall structure. It would be interesting to discover the mechanism by which these filamentous bacteria communicate with enterocytes in order that the latter provide a permanent attachment site for the bacterial cells.

The presence of filamentous segmented bacteria in the mouse intestinal tract leads to the upregulation of the murine gene (α[1-2]asialo GM1 fucosyltransferase) involved in fucosylation of the asialo GM1 glycolipid associated with enterocytes (Umesaki *et al.*, 1995). This phenomenon has also been reported in relation to colonization of ex-germfree mice with an anaerobic Gram-negative member of the intestinal microflora, *Bacteroides thetaiotaomicron* (Bry *et al.*, 1996). This bacterial species is able to utilize L-fucose, salvaged from intestinal glycoconjugates, as an energy source. The induction of fucosylation of glycoconjugates in the bowel of mice was shown to be dependent on the presence of a critical concentration of *Bacteroides* cells (10^6–10^7 CFU/ml) and on the ability of the bacteria to utilize L-fucose. A mutant strain, unable to utilize L-fucose, was less efficient at inducing fucosylation. In recent work, it has been observed that the linkage between L-fucose utilization and the system that is concerned with signalling enterocytes to induce fucosylation of intestinal glycoconjugates is mediated by the bacterial protein FucR (Hooper *et al.*, 1999). It is theorized that the *Bacteroides*, by influencing host biochemistry, ensure that L-fucose is constantly available as an energy source in a highly competitive environment. These kinds of studies open doors to an understanding of the complexity of microbe–host relationships and may provide insights into mammalian cell biology as well as evolutionary processes.

7. CONCLUSION

This chapter has recorded a variety of areas concerning the intestinal microflora that would benefit from the attentions of microbial physiologists. While the large-bowel ecosystem may seem an obscure and unpleasant field of

endeavour, the microbial complexity makes it a potentially fertile area for research. Moreover, the scientific surface of this neglected ecosystem has hardly been scratched!

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Primary Metabolism and its Control in Streptomycetes: A Most Unusual Group of Bacteria

David A. Hodgson

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

ABSTRACT

Streptomycetes are Gram-positive bacteria with a unique capacity for the production of a multitude of varied and complex secondary metabolites. They also have a complex life cycle including differentiation into at least three distinct cell types. Whilst much attention has been paid to the pathways and regulation of secondary metabolism, less has been paid to the pathways and the regulation of primary metabolism, which supplies the precursors. With the imminent completion of the total genome sequence of *Streptomyces coelicolor* A3(2), we need to understand the pathways of primary metabolism if we are to understand the role of newly discovered genes. This review is written as a contribution to supplying these wants. Streptomycetes inhabit soil, which, because of the high numbers of microbial competitors, is an oligotrophic environment. Soil nutrient levels reflect the fact that plant-derived material is the main nutrient input; i.e. it is carbon-rich and nitrogen- and phosphate-poor. Control of streptomycete primary metabolism reflects the nutrient availability. The variety and multiplicity of carbohydrate catabolic pathways reflects the variety and multiplicity of carbohydrates in the soil. This multiplicity of pathways has led to investment by streptomycetes in pathway-specific and global regulatory networks such as glucose repression. The mechanism of glucose repression is clearly different from that in other bacteria. Streptomycetes feed by secreting complexes of extracellular enzymes that break down plant cell walls to release nutrients. The induction of these enzyme complexes is often coordinated by inducers that bear no structural relation to the substrate or product of any particular enzyme in the complex; e.g. a product of xylan breakdown may induce cellulase production. Control of amino

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acid catabolism reflects the relative absence of nitrogen catabolites in soil. The cognate amino acid induces about half of the catabolic pathways and half are constitutive. There are reduced instances of global carbon and nitrogen catabolite control of amino acid catabolism, which again presumably reflects the relative rarity of the catabolites. There are few examples of feedback repression of amino acid biosynthesis. Again this is taken as a reflection of the oligotrophic nature of the streptomycete ecological niche. As amino acids are not present in the environment, streptomycetes have rarely invested in feedback repression. Exceptions to this generalization are the arginine and branched-chain amino acid pathways and some parts of the aromatic amino acid pathways which have regulatory systems similar to *Escherichia coli* and *Bacillus subtilis* and other copiotrophic bacteria.

ABBREVIATIONS

AAA	Aminoadipate
ABC	ATP-binding cassette
ADH	Alanine dehydrogenase
AEC	S-(2-aminoethyl)-L-cysteine
AHS	Acetohydroxy acid synthase
AIB	α-aminoisobutyric acid
AIR	5′-phosphoribosyl-5-aminoimidazole
AOAT	Alanine:2-oxoglutarate transaminase
ASase	Argininosuccinase
ASS	Argininosuccinate synthetase
DAHP	3-deoxy-D-arabino heptulosonate-7-phosphate
DAP	Diaminopimelate
DHOP	6,8-dihydroxy purine
EMP	Embden–Meyerhof–Parnas (pathway)
GDH	Glutamate dehydrogenase
GOAT	Glutamate:oxaloacetate transaminase
GOGAT	Glutamate synthase
GS	Gluamine synthetase
Hmp	Hexose monophosphate (pathway)
HPRT	Hypoxanthine phosphoribosyl-transferase
IMP	Inosine monophosphate
LAT	Lysine ε-aminotransferase
OAT	Ornithine aminotransferase
ORF	Open reading frame
P5C	Pyrroline-5-carboxylate
pABA	*p*-aminobenzoate
PEP	Phosph*enol*pyruvate
PTS	Phosphotransferase system
SAHC	S-adenosyl-homocysteine
SAICAR	5′-phosphoribosyl-4-(*N*-succinocarboxamide)-5-aminoimidazole
SAM	S-adenosyl-methionine
SAMP	Adenylosuccinate
TCA	Tricarboxylic acid (cycle)
TD	Threonine dehydratase
VDH	Valine dehydrogenase
XDH	Xanthine oxidase/dehydrogenase

1. INTRODUCTION

Streptomycetes are a group of high G+C ratio Gram-positive bacteria belonging to the Actinomycetes. Their property that most excites the interest of biologists is the ability to produce a very diverse range of metabolic products, some of which have important roles in medicine and veterinary science. The most common role is as antibiotics, but there are also other useful biological properties such as modulation of the immune system and enzyme inhibitors. The streptomycetes have proved one of the most useful sources of such secondary metabolites, and ten years ago it was reported that some 6000 antibiotics had been identified in the actinomycetes, of which 5000 were identified from streptomycetes (Goodfellow and O'Donnell, 1992). This was from a total of some 9000 known antibiotics from all organisms. About 500 new antibiotics are identified per year and still a very healthy proportion comes from the streptomycetes. This incredible diversity of biologically active natural products is reflected in the great importance of streptomycetes to the pharmaceutical industry, and natural product fermentation is the second largest income-generating biotechnological industry after brewing.

As well as the biotechnological interest in streptomycetes natural products, there is also a great deal of interest in streptomycete enzymes. Streptomycete glucose isomerase (more properly xylose isomerase) is used a great deal in the food industry for converting glucose to the 2.5-fold sweeter fructose. The streptomycete enzyme is robust and a heat-resistant form has been isolated from a thermophilic streptomycete.

Streptomycetes have a sophisticated developmental cycle, and recent discoveries of the underlying gene regulatory cascade are exciting interest in a number of academic research groups around the world (see below). The discovery of genetic conjugation in streptomycetes, specifically *Streptomyces coelicolor* A3(2), in the 1950s led to a very detailed understanding of streptomycete genetics which has proved of interest to the biotechnologist and the academic. The reader is recommended to study the recent excellent review of the development of *S. coelicolor* A3(2) genetics by Professor Sir David A. Hopwood (1999). These 40 years of study produced the surprising observations that streptomycete chromosomes are amongst the largest of bacteria, 8 megabases, that the chromosomes are linear and that there are very large linear, as well as the more conventional covalently closed circular, plasmids in many streptomycetes. The development of genetic and physical maps of the *S. coelicolor* A3(2) and an ordered cosmid encyclopaedia of the entire genome, with two gaps, has been exploited in the complete chromosome sequencing project at the Sanger Centre, Hinxton, Cambridge (http://www.sanger.ac.uk/Projects/S_coelicolor/). It is hoped the complete annotated genome sequence will be available by the end of 2000. From the

sequence already completed and annotated (about 64% as of March 2000) it is becoming clear there will be more genes than in the yeast *Saccharomyces cerevisiae*.

The aim of this review is to examine the nature and control of primary metabolism in streptomycetes. There is a large review literature on secondary metabolism and its control in streptomycetes, but very little on streptomycete primary metabolism. Such a review is timely because if we are ever to understand the control and functions of secondary metabolism, we must understand the nature and control of the metabolic processes that supply the precursors to secondary metabolism. Another reason for timeliness is the genome sequencing project, as annotation of the sequence will prove very difficult if we cannot provide information on the nature of primary metabolism and the sort of genes we would expect streptomycetes to have.

1.1. The Phylogeny of Streptomycetes

Streptomycetes belong to the sporoactinomycetes group in that they are Gram-positive, have a high G+C ratio (69–78%) and have complex colony morphology, which results in the production of arthrospores (see below). The spore-forming actinomycetes are split into a number of suprageneric sub-groups of which the streptomycetes is one. This group includes the genus *Streptomyces* and the genera *Intrasporangium*, *Kineosporia*, *Sporichthya* and *Streptoverticillium*. Members of these latter genera have very different morphological appearances, but the genera are so clustered because 16S RNA sequence has shown they are closely related and their cell walls are of the same chemotype (Goodfellow, 1989). Cell wall chemotype has proved a very useful means of taxonomic classification of the actinomycetes, which strongly correlated with sequence analysis-derived phylogenies. This review will be restricted mainly to the members of the genus *Streptomyces* as so little has been published on the primary metabolism of the members of the other genera.

Infrageneric *Streptomyces* classification has proved to be a difficult topic. Originally there were over 400 described *Streptomyces* species, and Williams *et al.* (1983), using classical numerical classification, resolved the species into 19 major cluster groups, designated with a capital letter (A–J), and 40 minor clusters, designated with an arabic numeral. The authors regarded the minor clusters as species. *Streptomyces coelicolor* A3(2) was a member of the largest major cluster group, A, and the minor group 21. This designation is used in parentheses throughout the review to show possible relatedness between individual species. *Species of uncertain taxonomic affiliation are marked with a question mark thus (?).*

1.2. The Ecological Niche of Streptomycetes

Streptomycetes are readily isolatable from soil, where their role appears to be to act as general saprophytes. They secrete extracellular enzymes and adsorb the soluble breakdown products of the interaction of those enzymes with insoluble polymers such as protein starch and cellulose. The source of most of this insoluble polymeric nutrient will be plants. It is clear, therefore, that the environment will be carbohydrate-rich but relatively nitrogen- and phosphate-poor. Being a member of the soil community, the streptomycetes will be in competition with a very large number of other microbes, including bacteria, fungi and protists, and so we might expect that soil is a nutrient-poor or oligotrophic environment.

In a review Williams (1985) made the statement: 'Overall assessments of the rates of energy input into the soil mass and the biomass of bacteria maintained within it suggest that soil is a grossly oligotrophic environment.' This may seem surprising considering the constant input of material into soil. A most important point Williams makes in the article is that 'while there is a great deal of nutrient input' the soil biomass is so great that it is very rapidly exhausted. While in the laboratory under rich nutrient conditions streptomycetes can have generation times as short as one hour, in woodland soil the generation time for a streptomycete was estimated as 1.7 days (Mayfield *et al.*, 1972; quoted in Williams, 1985). In his article Prof. Williams concluded that streptomycetes are facultative oligotrophs, i.e. they are capable of growing in both nutrient poor (oligotrophic) conditions and nutrient rich (copiotrophic) conditions. The following discussion of the control of streptomycete primary metabolism, in my opinion, makes most sense if we consider streptomycetes as facultative oligotrophs.

1.3. Pathogenic Streptomycetes

Plant pathogenic streptomycetes have been known for a very long time. A common pathogen is *S. scabies* (A3), which causes scab on root vegetables. The streptomycete penetrates the surface of the root and causes a warty proliferation of the dermis, which looks like a scab. This disease does not lead to loss of crop yield unless the growing conditions of the vegetable are very dry. However, the changes in the appearance can mean the farmer gets less money for a blemished product. It was once stated that all scab-causing streptomycetes belonged to the species *S. scabies* (A3). This view is no longer tenable (Bramwell *et al.*, 1998).

There is one species of *Streptomyces*, *S. somaliensis* (J70), that has been repeatedly found associated with actinomycetoma of animals, including man. A recent study has again suggested this pathogenic response may not be limited to the single species *S. somaliensis* (Steingrube *et al.*, 1997).

1.4. Autotrophic Streptomycetes

Ware and Painter (1955) reported a filamentous bacterium capable of growing on potassium cyanide as sole source of nitrogen and carbon on silica gel plates. Peptone and agar inhibited growth. The colonies formed aerial hyphae and small rod-shaped cells were released. This rather sounds like a nocardioform bacterium but, unfortunately, no further work on the strain was reported. Takamiya and Tubaki (1956) reported a streptomycete, *S. autotrophicus*, which was capable of growing on hydrogen, oxygen and carbon dioxide and a simple salts medium. The organism was reported to grow heterotrophically as well. Cochrane (1961) reviewed this report and drew the reader's attention to the work of Hirsch, who cast doubt on such reports because of the phenomenon of oligocarbophily, whereby cells can grow on hydrocarbon impurities in the air. The organism has recently been transferred to a new genus, as *Amycolata autotrophica* (Lechevalier *et al.*, 1986).

Kato *et al.* (1974) reported a streptomycete capable of growing on methanol as sole energy and carbon source. Later work by the group concerned the pathway of Cl fixation. However, cell wall and 16S rRNA sequence analysis of the organism has shown it to be a new species of the genus *Amycolatopsis*; i.e. *Amycolatopsis methanolica* (De Boer *et al.*, 1990).

Wainwright *et al.* (1984) reported the ability of a *Streptomyces* sp. to oxidize sulphur, thiosulphate and tetrathionate to sulphate. However, this strain could not fix carbon dioxide but it could grow oligocarbophilically. No evidence was presented which confirmed that energy could be harvested from this oxidation. Yagi *et al.* (1971) reported that the majority of streptomycetes could oxidize sulphur to thiosulphate. Of those that could not or could do so only poorly, *S. virginiae* and *S. lavendulae* were in the same minor cluster group (F6l) and *S. erythreus* has since been transferred to the genus *Saccharopolyspora*. Again no evidence was presented that implied that the bacteria could gain useful energy from the process. Kunert and Stransky (1988) demonstrated that the final product of keratin catabolism by a strain of *S. fradiae* (G68) was thiosulphate. Thiosulphate was also the product of a hydrogen sulphide-oxidizing enzyme purified from an uncharacterized streptomycete, *Streptomyces* sp. SH91, isolated from pig faeces compost (Ohta *et al.*, 1997).

A number of true autotrophic streptomycetes have been identified and characterized since the late 1980s. In all cases they gain ATP from oxidation of carbon monoxide (CO) and are moderate thermophiles. The first report was that of *Streptomyces* sp. G26 by Bell *et al.* (1987) and a very different streptomycete, *S. thermoautotrophicus* (?), was reported by Gadkari *et al.* (1990).

The taxonomic affiliation of *Streptomyces* sp. G26 has not yet been reported, but O'Donnell *et al.* (1993) isolated and characterized the taxonomy of 54 CO-utilizing streptomycetes and recently named two new species, *Streptomyces thermocarboxydovorans* and *Streptomyces thermocarboxydus*, as

representatives of the major cluster groups (Kim *et al.*, 1998). Surprisingly for a carboxydotrophic bacterium, *Streptomyces* sp. G26 could not grow on CO_2 as sole carbon and H_2 as sole energy source. However, more usually, and unlike *S. thermoautotrophicus*, it was a facultative chemolithotroph and could also grow on complex organic nutrient media. *Streptomyces* sp. G26 contains the enzymes of the Calvin CO_2 fixation cycle and a CO oxidoreductase. The latter enzyme was purified to 95% homogeneity and found to have a 'uniquely low specificity towards its oxidising substrate' (Bell *et al.*, 1988).

It appears that *S. thermoautotrophicus* is unique in being an obligate carboxydotrophic chemolithotroph. The CO oxidoreductase of *S. thermoautotrophicus* is unusual in its electron acceptor specificity (Gadkari *et al.*, 1990) and its failure to show any homology to the homologous class of CO oxidoreductases found in both Gram-positive and Gram-negative bacteria (Hugendieck and Meyer, 1992). It was also reported that *S. thermoautotrophicus* (?) could grow in the absence of any fixed nitrogen with a doubling-time of 10 h, and that $^{15}N_2$ could be incorporated into biomass (Gadkari *et al.*, 1992). $^{15}N_2$ incorporation was inhibited by addition of ammonium. There was evidence that an entirely novel fixation pathway was involved: there was no acetylene reduction; fixation was not inhibited by addition of acetylene, an inhibitor of nitrogenase; and there was no cross-hybridization with the highly concerned nitrogenase structural genes *nifH* and *nifKD*. However, there was evidence of hydrogen release during nitrogen fixation, which does occur during the action of nitrogenase. It was observed that the growth rate was not affected by growth on nitrogen or ammonium, although the growth yield was. This was a surprise, as nitrogen fixation requires a lot of ATP and reducing power, often limiting in autotrophs. Recently Ribbe *et al.* (1997) reported further on the dinitrogenase enzyme. Nitrogen fixation was coupled to CO oxidation by a molybdenum-containing CO dehydrogenase, which generated superoxide anion (O_2^-). The O_2^- was oxidized to O_2 by a manganese-containing O_2^- oxidoreductase and the electrons transferred directly to a molybdenum-iron-sulphur-containing dinitrogenase. The latter enzyme is unique in requiring oxygen and being resistant to hydrogen peroxide, whereas all other nitrogenases are exquisitely sensitive to oxygen and hydrogen peroxide.

1.5. The Developmental Programme of the Streptomycete Colony

One of the problems of a saprophytic life cycle, in which insoluble polymers are digested and the resulting monomers and oligomers are imported and used for biomass accumulation, is that this process takes time and relies on a high concentration of the digestive enzymes. One structure a number of microbes

have exploited to solve this problem is the substrate mycelium. The mycelium is made up of interdigitating substrate hyphae that are immobile, other than having movement by growth, and burrow into the insoluble food matrix, digesting it as they grow. The resultant mycelium is immobile, so the local concentration of the digestive enzymes is kept high. A corollary is that the local concentration of digestion products is also kept high. Another advantage of hyphal cells is that nutrient in soil will be present only in patches and individual hyphae can cross relatively large spaces within and between soil crumbs to allow colonization of distant patches of nutrient. The actinomycetes and the filamentous fungi have exploited this mycelial growth habit.

One disadvantage of the mycelial growth habit is that the bacterial colony is fixed and so some method of dispersal is needed, otherwise, once the nutrient patch has been exhausted death is the only result, unless the nutrient is fortuitously replaced. Some actinomycetes (e.g. *Nocardia* spp. and relations) have solved this problem by fragmentation of a pseudomycelium at the end of colony growth. Another alternative is to develop motile zoospores that can swim away to find a new niche once water has become plentiful (e.g. *Maduromyces* spp. and *Actinoplanes* spp.). *Streptomyces* spp. and many other actinomycetes have developed the ability to form hydrophobic exospores or arthrospores. These spores are synthesized as part of an aerial mycelium that overlays the substrate mycelium. This spore-bearing mycelium gives the colony a hairy appearance. Once a colony is washed with water the hydrophobic spores are broadcast owing to surface tension. Filamentous fungi (e.g. *Penicillium* spp.) also use the strategy of exploiting hydrophobic non-motile spores and, ignoring size, *Penicillium* spp. colonies bear a superficial resemblance to streptomycete colonies. Streptomycete spores are not very resistant to environmental insults such as elevated temperature or pH extremes. They are more resistant to desiccation than are hyphal fragments, however. This probably reflects the biological role of streptomycete spores; i.e. as dispersal agents rather than resting stages (Hodgson, 1992).

The life cycle of streptomycetes is fairly straightforward: a spore will germinate and outgrowth leads to the formation of a germ tube. This germ tube continues to lengthen to form a primary substrate hypha. The hypha branches and growth continues at the tip of the branched hyphae. The hyphae grow into the food source to form a branched, compact substrate mycelium. Hyphae begin to grow vertically upward out of the centre of the substrate mycelium, and these aerial hyphae form the aerial mycelium, which endows the colony with a hairy appearance. There is direct evidence that the substrate hyphae lyse and the products of lysis are cannibalized by the growing aerial hyphae. The initially straight aerial hyphae begin to twist to form tight coils, and then a very regular synchronized cell division of the hypha occurs. The result of this cross wall synthesis is the formation of a compartment that has a single copy of the genome. These compartments lay down specific cell wall layers, including a

hydrophobic cell sheaf, and round up to form ellipsoidal spores. A water droplet on the surface of the colony will then disperse the mature spores. Chater (1998) and Kelemen and Buttner (1998) have written reviews of the gene control pathways involved in streptomycete sporulation, but see Hodgson (1992) for a review of the more physiological aspects.

1.6. Streptomycete Secondary Metabolism

1.6.1. Primary versus Secondary Metabolism

Primary metabolism involves the catabolic and anabolic reactions that result in an increase in biomass; that is, the reactions that lead to the harnessing of energy and reducing power that in turn are used to synthesize the building blocks of proteins, nucleic acids, lipids and polysaccharide structural and storage materials. *Secondary metabolism* results in the synthesis of metabolites that have no further apparent function in metabolism. Streptomycetes have been studied extensively in the area of secondary metabolism, because of the obvious biotechnological importance of the products.

1.6.2. Streptomycete Secondary Metabolites

As well as the more renowned antibacterial antibiotics such as chloramphenicol, produced by *S. venezuelae* (A6), and streptomycin, produced by *S. griseus* (A15) and others, there are the herbicide bialaphos produced by *S. hygroscopicus* (A32), the immunosuppressive agent FK506 produced by *S. tsukabiensis* (?), and the β-lactamase inhibitor, clavulanic acid, produced by *S. clavuligerus* (J71). One of the more exotic streptomycete natural products is streptozocin, produced by *S. achromogenes* var. *streptozoticus*; this compound contains a dinitrogen bond that resembles that of the most potent alkylating agent *N*-methyl-*N′*-nitro-*N*-nitroso-guanidine. To assemble this dinitrogen bond, the streptomycete must generate nitrous acid, another potent mutagen, within its cytoplasm. How the bacterium avoids mutating its own DNA during streptozocin biosynthesis is not yet clear.

1.6.3. Control of Secondary Metabolism

At the same time as streptomycetes undergo morphological differentiation, they also undergo physiological differentiation and secondary metabolism is activated. This led to the proposal that one of the criteria for naming a compound as a secondary metabolite was that it was produced after the cell had

stopped growing. This aspect of the definition has often been challenged. However, it should it should be noted that when secondary metabolism is seen associated with the growth phase, the cells are often growing very slowly.

It is becoming a truism that all the genes involved in the biosynthesis of, and resistance to, a particular secondary metabolite are clustered at a single locus of the streptomycete genome and that they are very tightly regulated. This gene localization makes the coordinated regulation of the gene cluster simpler.

Secondary metabolite regulators have usually been identified as gene mutations, which lead either to overproduction of a metabolite (i.e. a repressor) or non-production of a metabolite (i.e. an activator). The former class has been reported far less frequently than the latter. Different classes of non-production mutant have been identified: (I) mutations that are pathway-specific and map to the biosynthetic gene locus; (II) mutations that block production of more than one secondary metabolite; and (III) mutations that block morphological differentiation in addition to secondary metabolism. Other classes of 'regulatory' genes were identified as multicopy suppressors or activators of secondary metabolism.

There is the danger of over-interpretation of a metabolite loss or overproduction phenotype. Loss of metabolite production could be due to loss of a gene necessary for expression that does not of itself control expression. A good example is the *bldA* gene of *S. coelicolor* A3(2) (A21) which encodes a tRNA gene that is required for translation of a number of pathway-specific regulators of metabolite production; i.e. *act*II orf4 and *redZ* for actinorhodin and undecylprodigiosin, respectively (Guthrie *et al.*, 1998). The *bldA* gene is part of the 'wiring' of the switch, rather than the switch itself. The *bld* mutants are very pleiotropic; in addition to loss of secondary metabolism they also do not produce an aerial mycelium and are deregulated for glucose repression of carbohydrate catabolism. They fall into class III of secondary metabolite non-production mutations.

There is currently a great deal of work to understand how the class I, class II and class III genes interact and how these genes interact with global regulatory networks such as stringent response and cAMP control (Bibb, 1996; Chakraburtty *et al.*, 1996; Chakraburtty and Bibb, 1997; Susstrunk *et al.*, 1998). A great deal of effort has been concentrated on the pathway-specific regulatory genes; their loss leads to an inability to express, or overexpression of, gene products involved in the biosynthetic pathway. As might be expected, these genes often encode DNA-binding proteins. RedZ, a gene product required for undecylprodigiosin gene (*red*) expression, turned out to be of the very common response regulator type but lacks the acid pocket and phosphate receptor aspartate residue characteristic of response regulators (Guthrie *et al.*, 1998). A number of pathway-specific regulatory genes have been found to

belong to a recently identified protein family with an unusual DNA binding domain with homology to the OmpR DNA-binding fold. This new class of protein, which includes RedD and ActII-orf4 of *S. coelicolor* (A3(2) (A21)), have been called SARPs – standing for ***Streptomyces*** **a**ntibiotic **r**egulatory **p**roteins (Wietzorrek and Bibb, 1997).

1.6.4. The Biological Roles of Secondary Metabolites

The real role of secondary metabolism in the biology of streptomycetes is not as obvious as it may appear at first glance. The genetic investment alone can be enormous. The current *S. coelicolor* A3(2) (A21) genome sequencing project reveals that the genes for production of the calcium-dependent antibiotic take up more than 1.1% of the chromosome. There is also the very great investment in genetic control machinery for these pathways. Therefore there has to be a role. I will spend some time discussing the conflicting hypotheses for the role of streptomycete secondary metabolism because our developing understanding of streptomycete primary metabolism control has a bearing on these hypotheses.

The correlation of initiation of secondary metabolism with development of the aerial mycelium led to the suggestion (Chater and Merrick, 1979) that the antibiotic aspect of secondary metabolites might have a role in streptomycete development. During lysis of the substrate mycelium there is the potential for microbial competitors to exploit the released cell sap. If secondary metabolites with antibiotic action were produced during substrate mycelium lysis they may inhibit any competitors of the aerial hyphae. This hypothesis is of the final product-selectionist school, if I may so term it. Another interesting hypothesis of this school was that of Davies (1990) who postulated that secondary metabolites might have acted as catalysts of macromolecular syntheses in ancient microbial ancestors before enzymes and ribozymes took their places. The review by Stone and Williams (1992) is a succinct encapsulation of the final product–selectionist school. Their main arguments are that secondary metabolite biosynthetic genes are clustered, coordinately regulated and tightly regulated, and the final products are too well fitted for their biological role for selection not to have had an important input into their evolution.

The main problems with the final product–selectionist hypotheses are: (1) not all secondary metabolites have a relevant biological function; (2) why do some streptomycetes have a plethora of secondary metabolites and others have few or none? and (3) how could the long and complex metabolic pathways of synthesis of most secondary metabolites have evolved? The first point is emphasized in immuno-modulatory secondary metabolites. How can such a biological role have exerted a selective advantage on the producing soil bacterium? The second point can be seen as a difficulty if we look at

streptomycetes, which produce a narrow-range antibiotic and a broader-range antibiotic. Surely the latter would always have a selective advantage over the former. The third point is a major flaw in the final product–selectionist hypotheses. An analogous evolutionary problem is that of evolving a bird wing: You cannot evolve a wing by a step-by-step process; unless the wing functions as a wing, i.e. confers flight, there is no selective advantage, so a wing has to be evolved in one step. This is a particular problem for bird evolution as the bird wing relies on the aerodynamic properties of feathers; this has led to the hypothesis that feathers were originally developed as insect-capturing devices whose aerodynamic potential was later exploited. The bird wing analogy is apt because the final product–selectionist hypothesis requires that something fairly close to the final product is required before selection can begin.

Evolution of primary metabolic pathways can be achieved using retro-evolution. This hypothesis was proposed by Horowitz (1945) and is predicated on the assumption that all the amino acids, nucleotide bases and enzyme cofactors etc. were available to the primitive cell in the prebiotic soup. Taking as an example the histidine pathway, if the primitive cells were consuming histidine, growth would be limited by the amount of histidine that could be produced by the prebiotic processes. If a cell developed an enzyme, presumably by gene duplication and mutagenesis, which could convert histidinol (also presumed to be in the prebiotic soup) to histidine, the cell would have an obvious selective advantage. If a descendant of this cell developed an enzyme that could convert imidazole acetol phosphate to histidinol phosphate, and there was a phosphatase that could dephosphorylate histidinol phosphate to histidinol, again a selective advantage would accrue. In this way, by recruiting enzymes to convert intermediates in the pathway backwards from the final product, the metabolic pathway eventually evolves such that the intermediates common to cell metabolism can act as pathway initiators. This hypothesis explains how there can be selection for each enzyme in the pathway one enzyme at a time.

The problem with evolving secondary metabolite biosynthetic pathways is that this selection does not exist in a step-by-step manner. Very often the direct precursor of an antibiotic has no antibiotic activity. In secondary metabolic pathways in which there are tens of enzymes, it is impossible to find conditions to select for each individual forward step. When pathways exist that produce compounds that are then assembled into a final compound, the hypothesis fails, as in the case of streptomycin, which is assembled from three novel sugars each produced by a novel pathway. The problem is that evolution cannot look forward. Once a minimally useful compound has been found, the final product–selectionist hypotheses explain how it can be refined into something more useful and then retained.

One possible way out of this apparent paradox is to examine some of the earlier ideas about secondary metabolism. Originally 'secondary metabolism'

was coined to explain observations in botany. It was found that many plants generated compounds from metabolism that were not converted further and that did not have an obvious role in the biology of the plant. For example, quinine produced by the cinchona tree is a very effective antimalarial compound but it is difficult to see how this benefits the producer. When similar compounds were found in fungi, the secondary metabolism was extended to include mycology and so on. These compounds were looked upon as products of 'overflow' metabolism; i.e. uncontrolled metabolism led to the production of compounds that then might be found by physicians to have some use. The problem with this hypothesis is that we know now that secondary metabolism is very tightly controlled. The hypothesis was altered to include the idea that the process of secondary metabolism was important to the cell rather than to the products of that metabolism. Under conditions of unbalanced growth when, for example, a major metabolite such as phosphate was missing, secondary metabolism could be induced to keep the major primary metabolic pathways 'ticking over' until the missing metabolite could be resupplied (Bu'Lock, 1961). It is very simple to unite this overflow metabolism hypothesis and the final product–selectionist hypotheses by stating that evolution of the initial part of a secondary metabolic pathway is driven by a need for overflow metabolism, and that products thus formed can then be subject to selection. Successful compounds would be retained and the genes for the enzymes involved could be corralled to a specific genetic locus over evolutionary time and coordinated regulation could be selected. The reason I discuss these two hypotheses is that our recent understanding of the control of streptomycete primary metabolism raises the possibility of overflow metabolism as a natural consequence of that control.

1.7. Problems of Studying Primary Metabolism in Streptomycetes

As discussed above, the streptomycete colony consists of a number of different cell types. Within any one of these cell types new metabolic pathways may be activated, and it may be difficult to distinguish between metabolites that are involved in differentiation and secondary metabolites. It might be claimed that in liquid culture the cells are exclusively of the substrate mycelium type. However, there have been reports that some streptomycetes can produce spores in liquid culture and that others can go through a microsporulation cycle (Hodgson, 1992).

The induction of metabolic differentiation is unlikely to be synchronous throughout even a liquid culture, which is a problem when studying streptomycete metabolism. It is exacerbated by the tendency of streptomycetes to form pellets and for growth to occur on the walls of the container above the

tidemark during liquid culture, especially in minimal media. The cells on the outside of the pellet will be in a different physiological state from those on the inside. Because liquid culture is the easiest way to obtain biomass for physiological studies, a number of methods have been used to minimize pellet growth. These include the use of baffles in the vessel, and the incorporation of polymeric molecules (e.g. Junlon and polyethylene glycol) in the medium to inhibit cell–cell adhesion (Hodgson, 1982; Hobbs *et al.*, 1989). Such actions, however, merely reduce the problem rather than abolish it. Microscopic examination of the cultures reveals that, unless the strain is one that fragments to an unusual degree, the cells form diffuse microcolonies, the cells at the centres of which are probably in a different physiological state from those on the outside.

Another approach is to filter out smaller microcolonies from larger ones in a culture in the hope that 'smaller' means younger (Sabater and Asensio, 1973a). Unfortunately the filtering process may break up 'old' large colonies. To be sure of obtaining young cells, spores can be spread on cellophane discs on solid media and harvested before cellular differentiation has occurred. Riesenberg and Bergter (1984) used freshly germinated and outgrown streptomycetes spores as a source of material. They demonstrated, by monitoring ATP, DNA, RNA and protein synthesis, and correlating these parameters with increase in mycelial length, that the cells underwent balanced growth for at least two cell doublings.

It is important to consider these problems because of the rapidly growing list of the number of streptomycetes in which isozymes of particular enzymes have been discovered. These isozymes have distinct properties and are expressed during different physiological states of the cell. Isozymes of enzymes important in primary metabolism have been shown to fall into two types: those that result in antibiotic autoimmunity; and those that are involved in secondary metabolism. An example of the former which is of direct relevance to primary metabolism was the report of Maurer *et al.* (1983) in which they announced the discovery of pentalenolactone-resistant and -sensitive isozymes of glyceraldehyde-3-phosphate dehydrogenase in *S. arenae* (A18), the producer of the antibiotic.

Two isozymes of kynurenine formamidase were found in *S. parvulus* (A12) (Brown *et al.*, 1986). One was involved in biosynthesis of actinomycin D, and was inducible; the other, which was constitutive, was postulated to have a role either in NAD synthesis or tryptophan breakdown. A similar situation was found in candicidin production. The enzyme *p*-aminobenzoic acid synthetase is an important enzyme in the synthesis of folic acid and the synthesis of candicidin *S. griseus* (A1B). The presence of two isozymes has been implied: one of which is regulated by phosphate and the aromatic amino acids and is involved in the synthesis of the antibiotic; and one that has not been detected but is presumably involved in primary metabolism (Gil *et al.*, 1985).

Another problem of studies of primary metabolism in streptomycetes is

the nature of strains used. If a comparison of primary metabolism and secondary metabolism is to be made it is essential that the production of the secondary metabolite be consistently inducible and be at such a level that it be easily assayable. This again may present problems. Many strains isolated directly from the environment may not produce much secondary metabolite. Therefore, there is the temptation to use strains that have been improved for secondary metabolite production and compare them with still more highly producing strains. The potential problem with this approach is that the improvement of secondary metabolite yield may have come about by the specific deregulation of the metabolic events one is hoping to study. There are some secondary metabolites that are produced at high levels in natural isolates, in particular pigments (Wright and Hopwood, 1976). Each case will depend on the research to be attempted, but the caveat should be borne in mind when considering primary metabolism studies on improved strains.

2. CARBON METABOLISM

2.1. The Central Pathways of Glucose Catabolism

2.1.1. Glycolysis

Cochrane (1961) reviewed the work on the pathways of glucose catabolism in streptomycetes. Evidence was presented that supported the use of the Embden–Meyerhof–Parnas (EMP) and hexosemonophosphate plus pentose phosphate (Hmp) pathways in *S. coelicolor* (A1A), *S. griseus* (A1B), *S. reticuli* (?) and *S. scabies* (A3). Isotope studies implied, at least for *S. griseus* (A1B), that the former was the more important pathway. Work by Dekleva and Strohl (1988a) expanded these observations to cover *Streptomyces* C5, *S. lividans* (A21) and *S. aureofaciens* (A14). These authors also report the absence of two key Eatner–Doudoroff pathway enzymes in these streptomycetes. Obanye *et al.* (1996) studied the EMP and Hmp pathways in *S. coelicolor* A3(2) (A21) and found the former to be active during exponential growth and the latter to be more active during the transition phase between exponential growth phase and stationary phase, when secondary metabolism was active. The essential glycolytic enzyme phosphofructokinase could not be detected in *S. antibioticus* (A3l) (Torbochkina and Dormidoshina, 1964; Torbochkina *et al.*, 1964). This work was confirmed and expanded by Salas *et al.* (1984). Isotope studies indicated glucose metabolism proceeded in the main via the Hmp pathway in the mycelium, whilst the EMP pathway was active in germinating spores.

One of the essential enzymes of the EMP pathway, phosphoglycerate mutase, has been purified and its gene cloned and sequenced from *S. coelicolor*

A3(2) (A21). This enzyme is responsible for the interconversion of 3-phosphoglycerate and 2-phosphoglycerate and had properties and sequence homology to the same enzyme from *Saccharomyces cerevisiae* (White *et al.*, 1992).

Another EMP pathway enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), has been studied in *S. arenae* (A18) and *S. aureofaciens* (A14). The enzyme was studied in *S. arenae* (A18) because there are two forms: one expressed during primary metabolism and one expressed as a resistance mechanism during the production of pentalenolactone, an inhibitor of the former isozyme (section 1.7). The pentalenolactone–insensitive form has been sequenced and been shown to be similar to other Gram-positive GAPDHs (Froehlich *et al.*, 1996). The gene for the *S. aureofaciens* (A14) GAPDH was identified as linked to an RNA polymerase sigma factor. The gene was shown to be induced by glucose and, in the absence of glucose, at the time of aerial mycelium formation (Kormanec *et al.*, 1997).

Phosphofructokinase is one of the main sites of regulation of the EMP pathway, and enzyme and its gene have been studied in *Streptomyces coelicolor* A3(2) (A21) (Alves *et al.*, 1997). The enzyme is ATP-dependent, in contrast to the enzyme from another actinomycete *Amycolatopsis methanolica*, which is pyrophosphate-dependent. The ATP-dependent enzyme was allosterically inhibited by phospho*enol*pyruvate (PEP). The stimulatory effect or otherwise of ADP and GDP was not reported.

The origin of the glucose-6-phosphate from glucose in streptomycetes has also received some attention. In many bacteria, glucose is phosphorylated as it is transported into the cell via the phospho*enol*pyruvate:carbohydrate:phosphotransferase system (PEP:PTS) (Saier, 1985). Sabater *et al.* (1972a) reported their inability to find a PEP:PTS system in *S. violaceoruber* (A21); however, they did find an ATP-dependent glucose kinase with an apparent K_m of 0.3 mM. Novotna and Hostalek (1985) confirmed the absence of a PEP:PTS system in *S. aureofaciens* (A14). Hodgson (1982) reported that mutants of *S. coelicolor* A3(2) (*S. violaceoruber* (A21)) lacking glucose kinase enzyme activity could nonetheless transport glucose, implying that a glucose PEP:PTS system was not present. Ikeda *et al.* (1984) further characterized the glucose kinase from this strain, confirming the absence of a PEP:PTS system for glucose. The enzyme had an apparent K_m of 0.27 mM for glucose. An ATP-dependent glucokinase activity was also identified in *S. clavuligerus* (J71). This was surprising, as the strain could not use glucose as sole energy or carbon source. The strain could however use starch and maltose, which implied that there was a lesion in glucose transport; i.e. maltose and other starch degradation products could get into the cell where they were broken down to glucose and phosphorylated. Recent work has confirmed this hypothesis (Garcia-Dominguez *et al.*, 1989). The presence of a polyphosphate-dependent glucose kinase was reported by Hostalek *et al.* (1976) in *S. aureofaciens* (A14). They found that the enzyme was present only after logarithmic growth, unlike the ATP-dependent kinase,

which was present only during logarithmic growth. Recent work has confirmed the presence of the 'minimal' PEP:PTS system for transport and phosphorylation of fructose in streptomycetes (section 2.2.3).

The glucose-6-phosphate dehydrogenase activity of *S. aureofaciens* (A14) has been purified by Neuzil *et al.* (1986 and 1988). They reported the discovery of two activities: an NAD-dependent one, which was sensitive to ATP, ADP, AMP, and phosphate inhibition; and an NADP-dependent one which was sensitive to neither adenylate compounds nor phosphate. Both activities were sensitive to reduced NAD and NADP. These results could be interpreted as evidence for the existence of two isozymes of the enzyme, the NADP-dependent form being used for generation of reducing power for anabolism, whilst the NAD-dependent form was used in the generation of ATP during glucose catabolism. An F_{420}-dependent glucose-6-phosphate dehydrogenase activity was found in *Mycobacterium* and *Nocardia* species but *not* in *Streptomyces* species (Purwantini *et al.*, 1997).

2.1.2. The Tricarboxylic Acid Cycle

The enzymes of the tricarboxylic acid (TCA) cycle have been studied in a number of streptomycetes because of their importance in the supply of precursors to secondary metabolism. Cochrane (1961) reviewed the evidence that the TCA cycle is complete and active in a number of streptomycetes. Dekleva and Strohl (1988a) demonstrated that a complete TCA cycle was also present in *Streptomyces* C5.

Hostalek, Vanek, Behal and co-workers have devoted a lot of time to studying the enzymes of this cycle in *S. aureofaciens* (A14). However, they did use improved strains in their studies. They reported (Hostalek *et al.*, 1969b) the presence of all the expected enzymes of the complete cycle and noted correlation of activity in different strains and ability to synthesize chlortetracycline. Hostalek (1969a) reported some of the characteristics of the citrate synthase. Like the enzyme from *E. coli*, ATP inhibited the enzyme and AMP stimulated it. However, unlike *E. coli*, reduced NAD had no affect. One, perhaps surprising, result of their studies (Tinterova *et al.*, 1969) was the discovery of isozymes of malate dehydrogenase showing different responses to oxaloacetic acid and requirements for magnesium. The recent purification and characterization of malate dehydrogenase from *S. aureofaciens* (A14) by Mikulasova *et al.* (1998) revealed a single enzyme with a strong preference for NADH and a more efficient back reaction, reduction of oxaloacetate, than forward reaction. Surprisingly, there was no evidence of product inhibition of malate oxidation; i.e. excess oxaloacetate did not inhibit.

2.1.3. Anaplerotic Reactions

The ability of many streptomycetes to grow on C2 compounds such as acetate and on aliphatic compounds implies the presence of active anaplerotic reactions. Without such reactions, the cell would soon run out of TCA intermediates for the synthesis of amino acids etc. In other bacteria the anaplerotic reactions include: (1) carboxylation of pyruvate by pyruvate carboxylase or carboxylation of PEP by PEP carboxykinase or PEP carboxylase to produce oxaloacetate; (2) generation of succinate and glyoxylate by splitting isocitrate with isocitrate lyase; and (3) generation of malate by condensation of glyoxylate and acetyl CoA with malate synthase.

In streptomycetes the enzyme involved in oxaloacetate formation has proved to be PEP carboxylase. This enzyme condenses PEP and CO_2 to form the acid and release orthophosphate. Vorisek *et al.* (1969) reported that the enzyme could be found in *S. aureofaciens* (A14) and that, like the *E. coli* enzyme, the activity was stimulated by acetyl CoA, as might be expected if the enzyme fulfilled an anaplerotic function. Nucleoside triphosphates, succinate, aspartate, and citrate inhibited the streptomycete enzyme, just like the *E. coli* enzyme. Unlike the *E. coli* enzyme, however, fructose 1,6-diphosphate did not stimulate it (Vorisek *et al.*, 1970).

PEP carboxylase was also found in *Streptomyces* C5 (Dekleva and Strohl, 1988b). The enzyme was purified 150-fold and AMP and fructose-1,6-biphosphate were found to stimulate its activity slightly. Oxaloacetate, malate, succinate, and aspartate strongly inhibited the enzyme, as did citrate and ATP, although to a lesser extent. The enzyme activity increased three-fold during the transition from primary to secondary metabolism. The same enzyme was also identified and purified from *S. coelicolor* A3(2) (A21). Partial amino acid sequence obtained from the purified enzyme was used to clone and overexpress the gene in *S. lividans* (A21) (Bramwell *et al.*, 1993). Little on the properties of the enzyme and the regulation of the gene were reported in this paper.

In *S. aureofaciens* (A14), malate synthase was found but not isocitrate lyase (Hostalek *et al.*, 1969b). Dekleva and Strohl (1988a) reported that *Streptomyces* C5, *S. lividans* (A21) and *S. aureofaciens* (A14) used acetate poorly and that the enzymes isocitrate lyase and malate synthase were not present. In the author's laboratory, *S. lividans* (A21) and *S. coelicolor* A3(2) (A21) grow on acetate, albeit poorly. However, these strains did grow well on Tween, a source of fatty acids, which, following fatty acid catabolism, is the same as growing on C2 compounds (C.P. Smith, personal communication). The relatively poor growth on acetate is probably related to weak acid poisoning. Isocitrate lyase and malate synthase were identified in *S. lividans* (A21) and *S. coelicolor* A3(2) (A21) during growth on Tween, but only the former when growing on acetate (I.S. Hunter, personal communication; Han

and Reynolds, 1997). Malate synthase has been identified in *S. arenae* (A18) grown on acetate or ethanol (Huettner *et al.*, 1997) and *S. clavuligerus* (J71) exposed to acetate (Chan and Sim, 1998). In neither case was there a report of isocitrate lyase activity.

The consistent absence of isocitrate lyase activity in streptomycetes growing on C2 compounds has led to the suggestion of an alternative route to glyoxylate in streptomycetes. When *S. collinus* (A18) was grown on acetate no isocitrate lyase was present. However, the enzyme was present when the streptomycete was grown on Tween. The proposed pathway of glyoxylate synthesis arose from the discovery of two genes that were essential for acetate utilization: *ccr,* which encodes crotonyl-coenzyme A (CoA) reductase, and *meaA*, which encodes a novel vitamin B_{12}-dependent mutase (Han and Reynolds, 1997). The authors proposed two pathways by which glyoxylate could be generated from acetyl-CoA. Both pathways would require propionyl-CoA carboxylase, which has been identified in *S. coelicolor* A3(2) (A21) (Bramwell *et al.*, 1996). The authors also demonstrated the activity of isobutyryl-CoA mutase and a methylmalonyl-CoA mutase and proposed an anaplerotic pathway whereby condensation of two acetyl-CoAs would lead to the formation of succinyl-CoA and hence succinate.

If either or both the proposed *S. collinus* (A18) glyoxylate-generating pathways exist and are common to streptomycetes, it would explain why malate synthase was present during acetate growth but not isocitrate lyase during C2 growth. The implication would be that growth on aliphatic compounds, such as fatty acids, involves the more common glyoxylate cycle with malate synthase and isocitrate lyase.

A number of enzymes that may be important in shuffling TCA cycle and EMP pathway intermediates have been reported in streptomycetes. Jechova *et al.* (1975) studied the malate enzyme (malate dehydrogenase, decarboxylating) which catalyses the conversion of malate to CO_2 and pyruvate and the reduction of NADP. This enzyme is used in other bacteria to generate NADPH for fatty acid synthesis. The enzyme from *S. aureofaciens* (A14) was repressed by about 50% when acetate was present in the medium. The activity of the enzyme directly paralleled the activity of fatty acid synthesis pathway in this strain, which was active only during logarithmic growth (Behal *et al.*, 1969). Redman and Hornemann (1980) investigated the ability of a number of actinomycetes to grow on pyruvate or alanine as sole carbon and energy source. Two streptomycetes *S. murayamaensis* (?), and *S. verticillatus* (?) contained the enzyme pyruvate, phosphate dikinase, that is responsible for the interconversion of pyruvate and PEP. The enzyme in the latter streptomycete was induced by growth on media containing alanine or pyruvate. This result implies that the enzyme is involved in gluconeogenesis. *S. achromogenes* var. *streptozoticus* (A19) did not contain the enzyme.

2.1.4. Acid Secretion

Streptomycetes are usually classified as obligately aerobic. Some strains have been reported to be able to grow microaerophically. There are two reports of dissimilatory nitrate and nitrite reduction in streptomycetes. The implication is that anaerobic respiration is possible with nitrate or nitrite as electron acceptor (Albrecht *et al.*, 1997; Shoun *et al.*, 1998) (see section 3.5.2). Albrecht *et al.* (1997) demonstrated that growth on nitrate under anaerobic conditions was impossible. So, the fact remains that streptomycetes seem to need oxygen. The majority of bacteria that use the EMP pathway or the hexosemonophosphate shunt can grow anaerobically on glucose and related sugars. They can harvest energy through substrate phosphorylation and regenerate reduced NAD by reduction of pyruvate to ethanol or lactate. Hockenhull *et al.* (1954) demonstrated that *S. griseus* (A1B) produced low levels of lactate during growth on glucose under a restricted aeration regime, which would imply that lactate dehydrogenase was present. We are left, therefore, with a conundrum: why can streptomycetes not grow anaerobically on glucose? Perhaps streptomycetes with lactate dehydrogenase, and/or pyruvate decarboxylase plus ethanol dehydrogenase, are sensitive to these waste products. Alternatively there could be a requirement for oxygen in some other essential process.

Whilst studying lactate production by *S. griseus* (A1B), Hockenhull *et al.* (1954) noted that during exponential growth pyruvate was excreted into the medium. Kannan and Rehacek (1970a) reported the excretion of TCA intermediates into the medium by *S. antibioticus* (A31). Since these initial reports quite a number of papers have been published on this phenomenon. Ahmed *et al.* (1984) reported acid secretion by older mycelium during nitrogen-limited growth in *S. venezuelae* (A6). They reported that during the transition to acid excretion the rate of glycolysis remained constant but there was loss of α-ketoglutarate dehydrogenase activity. They isolated a mutant that no longer excreted acid and in which the enzyme remained active in older mycelium. Unlike the case of *S. aureofaciens* (A14) (Doskocil *et al.*, 1959), the acids were not reabsorbed into the cell and used later, presumably because the α-ketoglutarate dehydrogenase activity remained low.

Surowitz and Pfister (1985a) examined acid secretion in *S. alboniger* (A1B) cells growing on glucose. This strain had been shown by Redshaw *et al.* (1976) to secrete organic acids during growth on glucose. These acids inhibited aerial mycelium formation, but this inhibition could be reversed by the addition of adenine. Surowitz and Pfister showed that the acid produced was pyruvate. They also showed, contrary to the observation in *S. venezuelae*, that the enzymes of the TCA cycle were not affected by growth on glucose; however, the rate of glycolysis increased. The lack of balance between glycolysis and the TCA cycle was responsible for the acid excretion. This imbalance was

corrected by the addition of adenine. The authors stated that they had not achieved conditions that led to reabsorption and use of the acid at a later stage.

Dekleva and Strohl (1987) reported the acidogenesis of *S. peuceticus*. In contradiction to all previous reports, this strain produced large amounts of acid only in the stationary phase, and then only in some cases and not others. All cultures produced low levels of pyruvate and α-ketoglutarate, however. Again, the excess acid was reabsorbed for reuse. These authors also reported that *S. lividans* and *S. coelicolor* A3(2) (A21) did not produce acid under their conditions. Conditions have been found for excretion of pyruvate and α-ketoglutarate and reabsorption of the former in *S. coelicolor* A3(2) (A21) and *S. lividans* (A21) (G. Hobbs and S. Oliver, personal communication; Madden *et al.*, 1996).

Why should some streptomycetes secrete acid? In at least some cases, it is clear that acid secretion is a result of imbalance between glycolysis and the TCA cycle. Some strains appear to shut down the TCA cycle, which may be a consequence of ageing, possibly a step in the shift from primary to secondary metabolism. The inability of some strains to stimulate the TCA cycle whilst stimulating glycolysis might be a consequence of the mode of gene regulation in that organism. Acidogenesis might be a stopgap method of dealing with excess glucose. Energy could be gained by substrate phosphorylation, and the product of glycolysis, in this case pyruvate, could be secreted into the medium for use later when the glucose supply was exhausted. Presumably more energy could be trapped and reduced NAD reoxidized by the action of the terminal respiratory pathway.

2.2. Peripheral Carbon Catabolic Pathways

It is not the intention of this section to be a comprehensive survey of all the carbohydrate catabolic enzymes and permeases reported to be made by streptomycetes, nor to discuss their industrial importance. It is rather the intention to discuss what we know of the regulation of such enzymes and their importance in the supply of materials for catabolism and anabolism of the cell. Unfortunately, as with much else concerning our knowledge of the streptomycetes, the importance industry may attach to an enzyme is often not reflected in our understanding of the biological significance of that enzyme to the organism. However, the more we understand about the synthesis and regulation of extracellular enzymes, the more the industrial scientist can exploit that knowledge.

Several terms require definition. These are inducers, repressors, inhibitors, and inducer exclusion. Inducers are low-molecular-weight compounds, which elicit the *de novo* synthesis of new proteins. This can occur at the level of transcription or translation. Repressors are low-molecular-weight compounds,

which stop the inductive effects of inducers when both of them are present at the same time. In this case the term 'repressor' should not be confused with DNA binding protein repressors, such as lacI. It should be noted that no particular molecular mechanism of repression is implied by the use of this term. The effect may be direct (e.g. at the level of transcription or translation) or indirect (e.g. via inducer exclusion). The term 'inhibitor' refers to compounds that decrease the action of a protein. Again the mechanism of action is not implicit in the term. It may be, for example, via allosteric interaction or by covalent modification. The term 'inducer exclusion' (Magasanik, 1970) refers to a particular mechanism of repression, namely, the inhibition of the cells' ability to import an inducer.

2.2.1. Complex Carbohydrate-Degrading Enzymes

Table 1 lists the reports concerning regulation of streptomycete polysaccharide-degrading enzymes. In all cases, these enzymes are extracellular enzymes, owing to the complexity of their substrates. Some of these enzymes have been used as models for assessing the nature of enzyme export machinery in streptomycetes.

2.2.1.1. a-Amylases These are enzymes that catalyse the cleavage of the α-1,4 linkage between glucose units within the dextran component of starch. The early work of Simpson and McCoy (1953) showed an interesting difference between streptomycetes in the way they regulated α-amylase production. The majority was induced by 'starch' – presumably a breakdown product of starch other than maltose – an exception being *S. microflavus* (A23) where maltose was the better inducer. Studies on a thermophilic streptomycete, *S. thermoviolaceus* (C45), revealed the presence of an α-amylase that was induced by maltotriose, maltotetraose and maltopentaose but not by maltose (Bahri and Ward, 1990).

In *S. hygroscopicus* (A32) α-amylase was reported to be of the type more readily induced by dextrin and starch. The authors (Grafe *et al.*, 1986) also reported that it was weakly repressed, not by the more usual glucose (see below) but instead by amino acids. This amino acid repressive effect was also seen in the maltase in this strain and in the maltase of *S. venezuelae* (Table 1). Exploiting a technique of mutant selection under continuous culture, these authors reported the isolation of a mutant that produced constitutively 20-fold more α-amylase than the uninduced wild type. This mutant produced a further 1.5-fold increase in enzyme production upon addition of an inducer. It had lost the two-fold repression of enzyme by amino acids seen in the wild type. A second mutant had lost the amino acid repressive effect but still required starch to be present to ensure efficient production.

Table 1 Regulation of polysaccharidase production in streptomycetes.

Enzyme	Inducer	Repressor	Strain	Reference
α-amylase (α-1,4-endoglucanase)	Starch, maltose	?	*S. cellulosae* (A13) *S. diastaticus* (A19) *S. griseus* (A15) 'Thermophile'	Simpson and McCoy, 1953
	Maltose, starch	?	*S. microflavus* (A23)	
	Dextrin Starch Maltose	Urea Aspartate Arginine Alanine Glycine	*S. hygroscopicus* (A32)	Grafe *et al.*, 1986
	Starch	Glucose Glycerol	*S. kanamyceticus* (B42)	Flores *et al.*, 1993
	Maltose maltodextrin	Mannitol	*S. limosus* (A1A)	Virolle and Bibb, 1988
	Maltotriose Maltotetraose Maltopentaose	Mannitol	*S. thermoviolaceus* (C45)	Bahri and Ward, 1990
	Maltose	Glucose	*S. venezuelae* (A6)	Virolle *et al.*, 1988
	Maltose	Glucose	*Streptomyces* sp. IMP2627	McMahon, *et al.*, 1997
α-glucanase (α-1,3-endoglucanase)	Glucan	?	*S. chartreusis* (A18)	Inoue *et al.*, 1975
	Glucan	?	*S. werraensis* (A12)	Takehara *et al.*, 1981

Table 1 *cont.*

Enzyme	Inducer	Repressor	Strain	Reference
α-glucan, mutan	Dextran	?	*Streptomyces* sp. KI-18	Imai *et al.*, 1977a
Cellulase (β-1,4-endoglucanase)	Xylan, cellulose	?	*S. flavogriseus* (A1C)	Kluepfel and Ishaque, 1982 MacKenzie *et al.*, 1987
	Cellobiose, Avicel	Glucose	*S. halstedii* (A1C)	Fernandez-Abalos *et al.*, 1997
	Xylan	Cellobiose	*S. lividans* (A21)	Kluepfel *et al.*, 1986 Shareck *et al.*, 1987
	Cellulose, xylan	?	*S. olivochromogenes* (A19)	MacKenzie *et al.*, 1987
Cellulase	Glucose Avicel Cellobiose Glycerol	Fructose	*S. reticuli* (?)	Walter and Schrempf, 1996a
	Straw, CMC, DMHC lignin, syringate	Glycerol	*Streptomyces* sp. EC1	Godden *et al.*, 1989
Cellulase CMCase	β-1,3-1,4-glucan CMC, β-1,3-1,4-glucan	?	*Streptomyces* sp. QM-B814	Perez-Pons *et al.*, 1994
Laminarinase (β-1,3-glucanase)	β-1,3-1,6-glucan gentiobiose	Glucose Glycerol	Thermophilic streptomycete	Lilley and Bull, 1974
	Laminarin	?	*Streptomyces* sp. QM-B814	Perez-Pons *et al.*, 1994
Xylanase (β-1,4-endoxylanase)	Xylan, xda	?	*S. flavogriseus* (A1C)	Kluepfel and Ishaque, 1982 MacKenzie *et al.*, 1987

Table 1 cont.

Enzyme	Inducer	Repressor	Strain	Reference
	Xylan, xylobiose to xylopentaose	Glucose	*S. lividans* (A21)	Vats-Mehta *et al.*, 1990 Arhin *et al.*, 1994
	Xda, xylan	?	*S .olivochromogenes* (A19)	MacKenzie *et al.*, 1987
	Xylan	?	*Streptomyces* sp.	Park and Toma, 1974
	Syringate, straw, xylan, lignin, DMC, CMC	Glycerol	*Streptomyces* sp. EC1	Godden *et al.*, 1989
Arabinofuranosidase	Xylan Xda, xylan	? ?	*S. flavogriseus* (A1C) *S. olivochromogenes* (A19)	MacKenzie *et al.*, 1987
4-O-methyl glucuronidase	Xda, xylan Xylan, xda	? ?	*S. flavogriseus* (A1C) *S. olivochromogenes* (A19)	MacKenzie *et al.*, 1987
Acetyl esterase	xda, cellulose, xylan xda	? ?	*S. flavogriseus* (A1C) *S. olivochromogenes* (A19)	MacKenzie *et al.*, 1987
Acetyl xylan esterase	Xda Xda	? ?	*S. flavogriseus* (A1C) *S. olivochromogenes* (A19)	MacKenzie *et al.*, 1987
Ferulate esterase	Xda Xylan	? ?	*S. flavogriseus* (A1C) *S. olivochromogenes* (A19)	MacKenzie *et al.*, 1987
Agarase (β-1,4-endo-galactosidase)	Agar hydrolysate	Glucose, mannose, cellobiose, rhamnose	*S. coelicolor* A3(2) (A21)	Hodgson and Chater, 1981 Bibb *et al.*, 1987

Table 1 *cont.*

Enzyme	Inducer	Repressor	Strain	Reference
Mannanse (β-1,4-endomannase)	Locust bean gum	?	*S. olivochromogenes* (A19)	Ratto and Poutanen, 1988
Chitanase (β-1,4-endoacetyl-glutaminase)	Chitin	Glucose	*S. lividans* (A21)	Miyashita *et al.*, 1991
	Chitin	Glucose	*S. plicatus* (A12) in *S. lividans* (A21)	Robbins *et al.*, 1992

CMC = carboxymethyl cellulose; DMC = 3,5-dimethoxy-cinnamate; DMHC = 3,5-dimethoxy-4-hydroxycinnamate; xda = deacylated xylan.

The α-amylase of *S. limosus* (A1A) was shown to be maltose-induced and mannitol-repressed (Table 1). This was a surprise, as glucose is the more usual repressing carbohydrate in streptomycetes (see below). When the gene encoding production of the enzyme was inserted into *S. lividans* and *S. coelicolor* A3(2) (A21), glucose and not mannitol repressed the enzyme. The mechanism of glucose repression in this latter strain has been shown to require the action of glucose kinase, and the repression of the *S. limosus* enzyme was also dependent on glucose kinase. The *S. limosus* enzyme was induced by maltotriose and subject to growth phase control in the *S. lividans* background (Virolle and Gagnat, 1994). Bahri and Ward (1990) reported that the α-amylase of *S. thermoviolaceus* (C45) was repressed by mannitol rather than glucose, but in this instance the cloned gene was not regulated by a carbon source in *S. lividans* (A21). Both induction and repression occurred at the level of transcription.

Sequence analysis of *S. limosus* showed that there was greater homology to mouse and *Drosophila* α-amylases than to those of plants, bacteria or fungi. This unexpected homology is also seen in the other streptomycete α-amylases that have been sequenced, namely, *S. hygroscopicus* (A32) (Hoshiko *et al.*, 1987), *S. venezuelae* (A6) (Virolle *et al.*, 1988) and *S. griseus* (A1B) (Vigal *et al.*, 1991). Nothing has been reported concerning the inducers or repressors of the *S. hygroscopicus* enzyme. In contrast, the *S. venezuelae* and *S. griseus* (A1B) enzymes are maltose-induced and starch-induced, respectively, and glucose-repressed, again at the level of transcription, both in the parental species and after cloning of the genes into *S. coelicolor* A3(2) (A21) or *S. lividans* (A21). In the *S. limosus* and *S. venezuelae* α-amylase genes, a consensus sequence similar to that found in the enteric maltose-regulated promoters was found to overlap the –35 regions of their promoters. There were other similarities upstream of the promoters (Virolle *et al.*, 1988).

S. lividans has always been treated as having no α-amylases. However, so far three genes, *amlA*, *amlB* and *amlC*, have been identified, whose products have significant identity to streptomycete α-amylases (Yin *et al.* 1997, 1998). Expression in *S. lividans* has been detected only when *amlB* and *amlC* were present on plasmids. Maltose or starch induced *AmlB*. *AmlC* was induced only by starch. Glucose repression was not tested.

2.2.1.2. Cellulases. Cellulose is a polysaccharide of glucose residues linked together by β-1,4 linkages of high tensile strength. Cellulose is the most abundant organic compound in the biosphere and so its degradation is of major ecological importance. There is some confusion in the literature. Enzymes have been called cellulases that are not capable of degrading crystalline cellulose but can degrade carboxy-methyl-cellulose. The more proper name for such enzymes is β-1,4-endoglucosidases and the term 'cellulase' should be retained for those enzymes capable of degrading crystalline cellulose.

Cellulose rarely occurs in nature as a pure polysaccharide. Usually it is in a complex with hemicellulose, which comprises mainly xylan, a β-1,4 linked xylose polymer which is heavily modified (see below), and lignin, a complex amorphous polymer of aromatic groups. Recently, a great deal of attention has been focused on cellulose degradation and the complex of enzymes that streptomycetes produce to achieve that degradation. It should be noted that lignin degradation does not yield products for further catabolism; rather ligninases are more important as a means of gaining assess to polymers that yield catabolites. As well as the endoglucanase, which cuts β-1,4 glycosidic bonds within the main body of the cellulose, an exo-glucanase has been identified in *S. flavogriseus* (A1C) that clips off cellobiose – the β-1,4 linked disaccharide of glucose (MacKenzie *et al.*, 1984). Alas, the regulation of this enzyme was not reported.

As well as the production of cellulose-degrading enzymes, specific cellobiases have also been reported (see below). So, in addition to the potential coordinate induction of cellulases, xylanases and ligninases, there is also evidence that there is coordinate induction of the catabolic pathways and permeases of the products of the action of the polymer-degrading enzymes.

In all cases (Table 1) the most efficient inducing agent was a complex polysaccharide. Presumably, a breakdown product, which could be transported into the cell, was the true inducer. In a number of cases, the preferred cellulase inducer was xylan rather than cellulose! In the most comprehensive study of induction, Godden *et al.* (1989) demonstrated that straw was the most efficient inducer when compared with any of the purified components tried, possibly reflecting a synergistic interaction of a number of inducers derived from various components of the straw. It is particularly interesting that a component of lignin, 3,5-dimethoxy-4-hydroxycinnamic acid, was an efficient inducer of the cellulase but not the xylanase.

The most comprehensive survey of cellulase production was performed on a collection of 40 uncharacterized streptomycetes isolated from soil (Skowronska, 1977a, b). In each case the effect was noted of various carbohydrates and amino acids on endo-cellulolytic and the exo-cellulloytic capabilities of liquid media cultures supplied with cellulose as the only carbon source, save the potential repressor or stimulator. Amino acids – cysteine, serine, threonine, phenylalanine and tryptophan – tended to be repressors of both the exo- and the endo- activities. However, in one case a strain was stimulated to produce both exo- and endo-cellulase on all the amino acids tested. The overall tendency of amino acids to repress the cellulases was reminiscent of the repression of the α-amylase in *S. hygroscopicus* (A32) (Table 1). Cellobiose stimulated cellulase production in the majority of cases, although in about a quarter of the strains tested the cellulases were repressed. The cellulases of about half the strains were repressed by glucose, albeit to a low level. In one case glucose stimulated exo-cellulase production greater than two-fold.

Xylose and mannose stimulated cellulase in about half, and repressed cellulase in the other strains. It is not clear whether stimulation or repression was correlated with the biomass accumulation rate.

Schrempf and her colleagues have extensively studied crystalline cellulose and xylan catabolism in *S. reticuli* (?). The active cellulase was processed by a specific protease into two fragments. The biological role of this processing is not understood (Schrempf and Walter, 1995). Avicel, a microcrystalline cellulose preparation (but none of the wide range of soluble cellulose products tested) induced the cellulase. Glucose, fructose, cellobiose and glycerol exerted repression, and it was noted that poorly buffered media became acidified – which inhibited enzyme activity and in part explained some of the 'repressive' effect of these carbohydrates. When the cloned enzyme was expressed in *S. coelicolor* A3(2) (A21), the repressive effects of glucose and glycerol were implied to have different mechanisms (Walter and Schrempf, 1996a). Evidence was presented for an activator and a repressor to be involved in expression of the cellulase gene (Walter and Schrempf, 1996b).

With regard to repressors of cellulases, Shareck *et al.* (1987) assert that cellobiose is a common repressor of endoglucanase biosynthesis in streptomycetes. Godden *et al.* (1989) clearly demonstrated the glycerol repression of cellulase production. Fernandez-Abalos *et al.* (1997) reported the identification of a putative cellobiose regulator region upstream of a cellobiose- and cellulose-induced, glucose-repressed type-B cellulase gene in *S. halstedii* (A1C).

2.2.1.3. Other Glucanases. α-1,3 and β-1,3 glucanases have been isolated from streptomycetes. In all cases (Table 1), the enzymes were induced by the substrate. The greater the proportion of α-1,3 linkages in the substrate, the higher the affinity of the enzyme for the substrate (Imai *et al.*, 1977a, b). This was also reflected in the inducing potential of a polysaccharide. The lower the proportion of α-1,3 glycosidic linkages, the lower was the efficiency of a substance as an inducer. In contrast, Lilley and Bull (1974) found that the β-1,6 linkage containing disaccharide gentiobiose was an efficient inducer of the β-1,3 glucanase of their thermophilic streptomycete. Glycerol was a repressor of the enzyme, although not as good as glucose.

2.2.1.4. Xylan-Degrading Enzymes. Xylan is a complex polysaccharide consisting of a backbone of β-1,4 linked xylose subunits. The subunits can be heavily modified. As well as a basic endoxylanase, which cleaves the β-1,4 linkages, there are enzymes that demodify the xylose and break the oligosaccharides down to xylose. In all cases studied the endo-xylanases were inducible by xylan (Table 1). The most extensive study of the regulation of the xylan-degrading enzyme complex (MacKenzie *et al.*, 1987; Johnson *et al.*, 1988) demonstrated that the different components of the complex were induced by different inducers, and that *S. flavogriseus* and *S. olivochromogenes* xylan-

degrading complexes were regulated in different ways. The report (Godden *et al.*, 1989) that syringic acid, a component of lignin, was the best inducer of endo-xylanase suggests that the true inducers of extracellular enzymes may be structurally unrelated to substrate of the enzyme, being derived instead from a component that is often found in association with the substrate. The discovery that xylan induces cellulases supports this suggestion.

S. lividans (A21) produces three xylanases, and there is evidence that xylan breakdown products produced by one xylanase are modified by transglycosylation by a second. These compounds then act as the true inducers of the xylanases (Arhin *et al.*, 1994). When the cloned xylanase genes were present on multicopy vectors, they were expressed at high level in streptomycete hosts. However, the *S. lividans* xylanase B was still repressed by glucose (Vats-Mehta *et al.*, 1990).

2.2.1.5. Agarase. Agar is a complex polysaccharide consisting of a β-1,4-linked D-galactose-3,6-anhydro-L-galactose disaccharide, linked by α-1,3 bonds. The precursor of 3,6-anhydro-L-galactose, L-galactose-6-sulphate, is often found in the final polysaccharide, as are methylated L- and D-galactosides and pyruvalated D-galactose. The gene, *dagA*, which encodes an agarase that cleaves the β-1,4 linkages of the backbone, has been cloned from *S. coelicolor* A3(2) (A21) (Kendall and Cullum, 1984; Bibb *et al.*, 1987) and sequenced (Buttner *et al.*, 1987). Extensive analysis (Bibb *et al.*, 1987; Buttner *et al.*, 1987, 1988; Servin-Gonzalez *et al.*, 1994) has shown that the enzyme has a signal sequence which enables it to be exported. There are multiple promoters for the gene, all of which are induced by agar breakdown products and are subject to glucose repression. There is evidence that the agarase gene is controlled by a single repressor. Loss of the repressor leads to constitutive expression that is still subject to glucose repression. Different forms of RNA polymerase containing alternative sigma subunits transcribe from the promoters. The necessity for such a complex expression system is not clear. The fact that all the promoters tested are regulated in the same way implies that differential physiological regulation is not the reason for the complexity. One possible suggestion was that the different sigma factors are expressed at different times during the growth or development cycle. However, S1 nuclease analysis of the *dagA* promoters has shown this not to be the case (Servin-Gonzalez *et al.*, 1994).

2.2.1.6. Mannanase. Mannans consist of β-1,4-linked mannose chains with side branches. These branches often contain galactose residues. Takahashi *et al.* (1984) reported the purification of an endo-mannanase from an unclassified streptomycete capable of cleaving the mannan backbone into mannose, mannobiose and mannotriose. The enzyme could also cleave mannotriose to mannobiose and mannose. The mannobiose was not cleaved further. Ratto

and Poutanen (1988) reported a similar, inducible, enzyme from *S. olivochromogenes* (Table 1). However, they could not find mannobiase activity in the culture filtrate. Presumably, this enzyme was located within the cell.

2.2.1.7. Chitinases. Chitins are present in insect exoskeletons and fungal cell walls. They consist of β-1,4-linked acetylglucosamine residues. The streptomycetes are particularly rich in exo- and endo-chitinases and can utilize the products as nitrogen and carbon and energy sources (Robbins *et al.*, 1992). The enzymes of *S. plicatus* (A12) have been studied in some detail following cloning into *E. coli* and *S. lividans* (A21). The enzymes are induced by chitin in *S. lividans* (A21) and *S. plicatus* (A12) and are repressed by glucose, at least in *S. lividans* (A21) (Robbins *et al.*, 1992). Delic *et al.* (1992) reported the discovery of a directly repeated 12-base-pair sequence upstream of a number of chitinase genes, which when mutated led to loss of chitin dependence for expression and glucose repression. They also demonstrated the existence of protein, which bound to this region. The endogenous chitinases of *S. lividans* (A21) have been cloned also and shown to be induced by chitin and repressed by glucose and glycerol (Miyashita *et al.*, 1991).

2.2.2. Disaccharide Degrading Enzymes

Our knowledge of the regulation of disaccharidases of streptomycetes is summarized in Table 2. One major difference between the enzymes concerns their cellular or extracellular location. This variation in location might be expected to have consequences for the regulation of the enzyme.

2.2.2.1. Maltase. Chatterjee and Vining (1981) reported the induction of an α-1,4-glucosidase (maltase) by isomaltose and maltose in *S. venezuelae* (A6). They demonstrated that the induction could be halted by rifampicin, chloramphenicol and streptomycin, demonstrating that induction was at the level of transcription.

2.2.2.2. β-glucosidases. Chatterjee and Vining (1982b) discovered two β-glucosidases in *S. venezuelae* (A6). One was a cellobiose-induced, glucose-repressed enzyme capable of splitting cellobiose (cellobiase), whilst the other was a salicin-induced, acetate- and amino acid-repressed enzyme capable of hydrolysing aryl β-glucoside but not cellobiose. The presence of the two β-glucosidases in one strain, both capable of cleaving unnatural chromogenic substrates such as *p*-nitrophenyl β-D-glucopyranoside but both regulated in different ways, should be borne in mind when considering apparently contradictory results from other streptomycetes. The physiological role of the aryl β-glucosidase is not clear.

Regulation of the cellobiases of *S. flavogriseus* (A1C) and *Streptomyces* sp. CB-12 (Moldoveanu and Kluepfel, 1983) shows interesting contrasts. The *S. flavogriseus* (A1C) enzyme was glucose-induced whilst that of the second streptomycete was induced by cellobiose. The authors also claimed that the *Streptomyces* sp. CB-12 enzyme was glucose-repressed. However, in the experiment reported they did not add the inducer (cellobiose) to the same cells to which they added the potential repressor. Hence, it is possible to interpret the results as lack of induction rather than repression. The cellobiases of two uncharacterized streptomycetes were also found to be induced by cellobiose, and they retained this control when the genes encoding them were cloned into the heterologous host, *S. lividans* (A21) (Jaurin and Granstrom, 1989). Again the presence or absence of glucose repression cannot be assessed because inducer was not included when glucose was present.

The *S. granaticolor* (?) and *Streptomyces* sp. EC1 cellobiases (Table 2) were induced by cellobiose and repressed by glucose or glycerol, respectively. Ozaki and Yamada (1991) screened for bacteria that produced β-glucosidases that were resistant to glucose inhibition. They identified a *S. thermodiastaticus* (A1C) strain that produced three such intracellular enzymes. All three purified enzymes had β-glucosidase activities. There is evidence of intracellular and extracellular β-glucosidases in *S. reticuli* (?). They were induced in the presence of cellobiose, Avicel and glycerol. Nothing was reported with respect to repression. An intracellular β-glucosidase was purified and found to hydrolyse cellobiose preferentially, although there was decreasing activity on oligosaccharides up to cellohexaose. The enzyme was inhibited by glucose (Heupel *et al.*, 1993).

2.2.2.3. β-Galactosidases. Two types of β-galactosidases have been identified in streptomycetes: the galactose-inducible β-galactosidase which is often incapable of hydrolysing lactose, typified by that found in *S. violaceus* (A6); and the lactose-induced, lactose hydrolysing enzyme, typified by that found in *S. griseus* (A1B) and *S. venezuelae* (A6) (Table 2). Both types of enzyme can cleave chromatogenic β-galactosides (e.g. X-gal), but only the lactose-induced type has been shown to have a catabolic function. This sounds rather similar to the situation found with the β-glucosidases (section 2.2.2.2). Examples of both types of β-galactosidase were glucose-repressible.

X-gal-cleaving β-galactosidases have been found in both *S. coelicolor* A3(2) (A21) (King and Chater, 1986) and *S. lividans* (A21) (Burnett *et al.*, 1985). Neither strain could use lactose as the sole carbon source. The *S. lividans* enzyme was shown to be extracellular and has been used as an export vehicle (Eckhardt *et al.*, 1987). A β-galactosidase with uncharacterized substrate specificity, identified in *S. parvulus* (A12), was found to be galactose-inducible and preferentially repressed by amino acids rather than carbohydrates (Foster and Katz, 1981). This type of regulation is reminiscent of that seen with some maltases and cellobiases from other streptomycetes (Table 2).

Table 2 Regulation of disaccharidase production in streptomycetes

Enzyme	Inducer	Repressor	Strain	Reference
Maltase (α-1,4-glucosidase)	Starch, dextrin, maltose	Alanine Aspartate	*S. hygroscopicus* (A32)	Roth *et al.*, 1985, 1986 Grafe *et al.*, 1986
	?	Not amino acids	*S. noursei* (?)	
	Isomaltose, maltose starch, maltotriose dextrin	Glycine, serine asparagine, alanine, glutamate acetate	*S. venezuelae* (A6)	Chatterjee and Vining, 1981
Cellobiase (β-1,4-glucosidase)	Glucose Not cellobiose	?	*S. flavogriseus* (A1C)	Kluepfel and Ishaque, 1982 Moldoveanu and Kluepfel, 1983
	Cellobiose	?	*Streptomyces* sp. C-12	
	Cellobiose and glycerol	Glucose	*S. granaticolor* (?)	Jiresova *et al.*, 1987
	Cellobiose, Avicel glycerol	?	*S. reticuli* (?)	Heupel *et al.*, 1993
	Cellobiose	?	*S. thermodiastaticus* (A1C)	Ozaki and Yamada, 1991
Class I	Cellobiose	Glucose, acetate, amino acids	*S. venezuelae* (A6)	Chatterjee and Vining, 1982b
Class II	Salicin	Acetate, amino acids		
	Cellobiose, cellotriose, cellotetraose, CMC	Glycerol	*Streptomyces* sp. EC1	Godden *et al.*, 1989
	Cellobiose	Glucose	*Streptomyces* sp. QM-B814	Perez-Pons *et al.*, 1995
	Cellobiose		*Streptomyces* sp. UM-2 *Streptomyces* sp. UM-d	Jaurin and Granstrom, 1989

Table 2 cont.

Enzyme	Inducer	Repressor	Strain	Reference
Xylosidase (β-1,4-xylosidase)	Xylan Cellulose	?	*S. flavogriseus* (A1C)	Kluepfel and Ishaque, 1982
	Xylobiose Xylan	Glycerol	*Streptomyces* sp. EC1	Godden *et al.*, 1989
β-galactosidase (β-1,4-galactosidase)	Lactose	?	*S. griseus* (A15)	Dan and Szabo, 1973
	Glutamate Galactose Proline	Aspartate	*S. parvulus* (A12)	Foster and Katz, 1981
	Lactose, galactose, galactosamine, Arabinose	Glucose Maltose Acetate	*S. venezuelae* (A6)	Chatterjee and Vining, 1982a
	Galactose Arabinose	Glucose, ribose gluconate, cellobiose, tryptophan	*S. violaceus* (A6)	Sanchez and Hardisson, 1979, 1980a, 1980b

Table 3 Inducible carbohydrate uptake in streptomycetes.

Enzyme	Inducer	Repressor	K_m	V_{max}	Strain	Reference
Arabinose	Arabinose	Glucose	?	?	*S. coelicolor* A3(2) (A21)	Hodgson, 1982
Cellobiose	Cellobiose	Glucose	0.5 μM	0.053[a]	*S. granaticolor* (?)	Jiresova *et al.*, 1987
	Cellobiose	?	400 μM	0.9[a]	*S. lividans* (A21)	Hurtubise *et al.*, 1995
	Cellobiose	?	4 μM	7[b]	*S. reticuli* (?)	Schloesser and Schrempf, 1996
Fructose	Fructose	Glucose	?	?	*S. antibioticus* (A31)	Salas and Hardisson, 1981
	Fructose		?	?	*S. coelicolor* A3(2) (A21) *S. lividans* (A21)	Titgemeyer *et al.*, 1995
	Fructose, mannitol	?	5 μM	8.0[a]	*S. venezuelae* (A6)	Tang, 1977
Galactose	Galactose	Glucose	?	?	*S. antibioticus* (A31)	Salas and Hardisson, 1981
Glycerol	Glycerol	Serine	14μM	?	*S. clavuligerus* (J71)	Minambres *et al.*, 1992
	Glycerol	Glucose	?	?	*S. coelicolor* A3(2) (A21)	Hindle and Smith, 1994
Maltose	Maltose	Glucose	?	?	*S. coelicolor* A3(2) (A21)	van Wezel *et al.*, 1997a

Table 3 *cont.*

Enzyme	Inducer	Repressor	K_m	V_{max}	Strain	Reference
Mannose	Mannose	Glucose	?	?	*S. alboniger* (A1B)	Surowitz and Pfister, 1985a
Sucrose	Sucrose	Glucose	?	?	*S. alboniger* (A1B)	Surowitz and Pfister, 1985a
Xylose	Xylose	?	200 μM	2.1[a]	*S. lividans* (A21)	Hurtubise *et al.*, 1995
Xylobiose	Xylobiose	?	100 μM	0.03[a]	*S. lividans* (A21)	Hurtubise *et al.*, 1995

[a] nmol min^{-1} (mg protein)$^{-1}$; [b] nmol min^{-1} (dry weight)$^{-1}$.

Table 4 Constitutive carbohydrate uptake in streptomycetes.

Enzyme	Inhibitor	K_m	V_{max}	Strain	Reference
Fructose	Glucose	47 mM	46.5[b]	*S. coelicolor* A3(2) (A21)	Hodgson, 1982
	None	3 mM	0.8[c]	*S. violaceoruber* (A21)	Sabater and Asensio, 1973a
Galactose	Glucose	?	?	*S. violaceus* (A6)	Sanchez and Hardisson, 1980a,b
	Glucose	6.1 mM	5.4[b]	*S. coelicolor* A3(2) (A21)	Hodgson, 1982
Glucose					
Type I	?	0.33 mM	0.95[b]	*S. aureofaciens* (A14)	Novotna *et al.*, 1985
Type II	?	1.6 mM	1.87[b]		
Type I	?	0.05 mM	0.19[b]	*S. clavuligerus* (J71) (*gut-1* mutant)	Garcia-Dominguez *et al.*, 1989
Type II	?	3.7 mM	1.0[b]		
	None	6.1 mM	?	*S. coelicolor* A3(2) (A21)	Hodgson, 1982; Button, 1985
Type I	?	0.12 mM	66[a]	*S. lividans* (A21)	Hurtubise *et al.*, 1995
Type II	?	6.2 mM	27[a]		
	Mannose	1 mM	0.5[c]	*S. violaceoruber* (A21)	Sabater and Asensio, 1973a
Mannose	Glucose	1.5 mM	0.8[c]	*S. violaceoruber* (A21)	Sabater and Asensio, 1973a

[a] nmol min^{-1} (mg protein)$^{-1}$; [b] nmol min^{-1} (dry weight)$^{-1}$; [c] nmol min-1 (wet weight)$^{-1}$.

2.2.3. Carbohydrate Uptake

Once complex polysaccharides have been broken down by the extracellular enzymes into oligo-, di- and monosaccharides, they can be taken into the cell. In some cases, oligo- and disaccharidases may be present inside and outside the cell. One imagines that it would be to the streptomycetes' advantage to be able to transport as complex a carbon and energy source as possible into the cell so as to deny them to any competitor.

As discussed above (section 2.1.1), attempts to find a PEP-coupled phosphotransferase system (PEP-PTS) for glucose transport and phosphorylation in streptomycetes have proved unsuccessful. However, Titgemeyer *et al.* (1995) have presented evidence for the presence of a PEP-PTS for fructose transport in *S. lividans* (A21), *S. coelicolor* A3(2) (A21) and *S. griseofuscus* (A12). The systems were inducible by fructose in the first two strains and constitutive in the third. Specific fructose transport was not assayed, but membrane-bound PEP-dependent phosphorylation of fructose was observed in addition to a soluble ATP-dependent fructose phosphorylation in *S. lividans* and *S. coelicolor* A3(2). There was already overwhelming evidence of an ATP-dependent fructose kinase in the closely related *S. violaceoruber* (A21) (Sabater *et al.*, 1972b; see also section 2.2.4.4). The constitutive PEP-dependent and ATP-dependent fructose phosphorylation activities found in *S. griseofuscus* were lower than in the other two streptomycetes, and there was only weak evidence for a membrane-bound PEP-dependent phosphorylation of fructose in this streptomycete. No PEP-dependent glucose phosphorylation activity was found in any of the streptomycetes, nor were the HPr (Ser) kinase or HPr (Ser-P) phosphatase activities found. The latter activities are central to PEP-PTS control of catabolite repression in low GC Gram-positive bacteria. We must conclude, therefore, that the PEP-PTS fructose transport system has no role in catabolite repression in streptomycetes (see section 2.4).

A number of carbohydrate uptake systems have been identified and studied in streptomycetes. They are classified into two types: inducible (Table 3) and the constitutive (Table 4). The inducible carbohydrate transport systems were generally induced by the substrate. Glucose repressed a number of inducible transport systems and inhibited a number of constitutive transport systems, which raises the possibility that the mode of action of glucose repression is via inducer exclusion; i.e. glucose inhibits the transport of the inducer into the cell (see section 2.4.5.2). In a number of cases, 'constitutive' uptake systems were found only in mycelia but not in spores. Glucose transport systems were present in *S. violaceoruber* (A21) (Sabater and Asensio, 1973a) and *S. antibioticus* (A31) (Salas and Hardisson, 1981) spores. In the case of *S. violaceoruber* (A21) the germination process induces the 'constitutive' fructose and mannose.

Where substrate affinity (K_m) of streptomycete carbohydrate uptake systems

has been reported, a dichotomy is apparent. The inducible uptake systems show affinities similar to those seen for carbohydrate uptake in most other bacteria; i.e. within the µM range. However, the constitutive uptake systems have substrate affinities that are a thousand-fold, or more, lower in affinity. It is a pleasing congruence that the theoretical calculation of the K_m for glucose transport in *S. coelicolor* A3(2) (A21), 6.1 mM, agreed with the experimentally determined value for glucose transport by *S. lividans* (A21), 6.2 mM (Table 4).

It is interesting to note that no one has reported the isolation of a carbohydrate transport mutant using toxic sugar analogues such as 2-deoxyglucose (Hodgson, 1982) and 2-deoxygalactose (Kendall *et al.*, 1987). Perhaps there are multiple transport systems present, as observed for glucose in *S. aureofaciens* (A14) (Novotna *et al.*, 1985) and *S. lividans* (A21) (Table 4). The high-affinity glucose permease in *S. lividans* (A21) has a V_{max} of 66 nmol min^{-1} mg $(protein)^{-1}$. The equivalent value for the low-affinity system is 27 nmol min^{-1} mg $(protein)^{-1}$.

It appears that *S. clavuligerus* NRRL 3585 (J71) is a natural glucose transport mutant (Garcia-Dominguez *et al.*, 1989). This strain has the ability to grow on starch, maltose and glycerol, but not on glucose or galactose. As discussed above (section 2.1), it was demonstrated that the strain had an ATP-dependent glucokinase. A mutant, *gut1*, was isolated that could grow with glucose as sole energy and carbon source. The mutant also grew on galactose. The kinetics of glucose transport was examined in both wild type and mutant. Two transport systems were identified in both strains. A high-affinity, (5–6 µM) low-capacity (140–190 nmol min^{-1} mg (dry $weight)^{-1}$) transport was present in both strains. A low-affinity (3.7 mM) high-capacity (1.0 mmol min^{-1} mg (dry $weight)^{-1}$ transport system was present in the mutant that had higher affinity, but lower capacity, than the equivalent system in the wild type (K_m = 12.5 mM and V_{max} = 2.5 mmol min^{-1} mg (dry $weight)^{-1}$). It is presumed that the changes in the kinetic parameters of the low-affinity transport system are responsible for the ability of the *gutl* mutant to use glucose as a carbon source.

There is now clear evidence that disaccharide transport in streptomycetes is via five component ABC permeases: maltose in *S. coelicolor* A3(2) (A21) (van Wezel *et al.*, 1997a,b); cellobiose/cellotriose in *S. reticuli* (?) (Schloesser and Schrempf, 1996; Schloesser *et al.*, 1997, 1999); and cellobiose, xylobiose and maltose in *S. lividans* (A21) (Hurtubise *et al.*, 1995; Schloesser *et al.*, 1997). A specific cellobiose-binding lipid-anchored protein has been identified in *S. reticuli* (?). This protein is equivalent to the sugar-binding proteins found in the periplasm of Gram-negative bacteria. As streptomycetes are Gram-positive, they do not have a periplasm and so such 'periplasmic' proteins have to be anchored by a fatty acid adduct, in this case palmitate (Schloesser and Schrempf, 1996). A gene product, MsiK, has been identified in *S. lividans* (A21) and *S. reticuli* (?) that is involved in the independent transport systems for cellobiose, xylobiose and maltose in the former streptomycete, and for

cellobiose in the latter streptomycete (Hurtubise *et al.*, 1995; Schloesser *et al.*, 1997). Loss of MsiK leads to loss of ability to transport all of the different disaccharides. The maltose-induced, glucose-repressed maltose transport genes, *malEFG*, of *S. coelicolor* A3(2) (A21) are subject to the control of a repressor, MalR. This repressor is related to the LacI-GalR family of repressors. Loss of *malE* led to loss of ability to transport maltose. Loss of MalR led to constitutive expression of *malEFG* and expression was no longer subject to glucose repression (van Wezel *et al.*, 1997a, b). The homologue of MalR in *S. lividans* (A21), Reg1, was shown to be required for repression of amylase and chitinase genes. Loss of Reg1 led to inducer-independent expression of the extracellular enzymes and loss of glucose repression (Nguyen *et al.*, 1997a).

2.2.4. *Inducible Intracellular Carbohydrate Catabolic Enzymes*

Over the last few years a number of carbohydrate catabolism genes have received detailed attention. Table 5 contains a summary of some of this work. The glycerol, xylose and galactose catabolism genes have been cloned and subjected to molecular analysis.

2.2.4.1. Glycerol Catabolism. *S. coelicolor* A3(2) (A21) (Seno and Chater, 1983), *S. lividans* (A21) and *S. griseus* (A1B) (Biro and Chater, 1987) catabolise glycerol via a glycerol-inducible, glucose-repressible glycerol transport facilitator (the *gylC* product), glycerol kinase (the *gylA* product) and glycerol-3-phosphate dehydrogenase (the *gylB* product). The operon is arranged as *gylCABX*, where *gylX* is of unknown function (Hindle and Smith, 1994). There is strong evidence that the true inducer of the operon is glycerol-3-phosphate, the product of glycerol kinase activity (Seno and Chater, 1983).

Upstream of the operon is *gylR* (formerly the '0.9-kb gene'), which encodes a 27 600 Mr protein with a helix-turn-helix domain characteristic of DNA-binding proteins (Smith and Chater, 1988a, b). Loss of this gene leads to constitutive expression of the operon; i.e. it is a repressor. Point mutations in the *gylR* gene that cause a Gyl^- phenotype must, therefore, be super-repressor mutations. Interestingly, the constitutively expressed operon in the $gylR^{\Delta}$ mutant is no longer subject to glucose repression (Hindle and Smith, 1994). The *gylR* region of *S. griseus* (A1B) has also been sequenced (Bolotin and Biro, 1990). The *gylR* gene showed 81% sequence identity to the *S. coelicolor* A3(2) (A21) *gylR* gene. This value shows agreement with that obtained from analysing the levels of hybridization and restriction site polymorphism between the two strains; i.e. about 87% (Biro and Chater, 1987). They also reported that the genes of *S. lividans* (A21) were 99% similar, and those of *S. parvulus* (A12) were 96% similar, to the *S. coelicolor* A3(2) genes.

Table 5 Regulation of carbohydrate catabolism in streptomycetes.

Enzyme	Inducer	Repressor	Strain	Reference
Arabinose	Arabinose	Glucose	*S. coelicolor* A3(2) (A21)	Hodgson, 1982
Fructose	Fructose	Glucose	*S. coelicolor* A3(2) (A21)	Hodgson, 1982
	Fructose	?	*S. violaceoruber* (A21)	Sabater *et al.*, 1972a
Galactose	Galactose	Glucose	*S. coelicolor* A3(2) (A21)	Hodgson, 1982
	Galactose	Glucose	*S. lividans* (A21)	Fornwald *et al.*, 1987
Glycerol	Glycerol	Glucose	*S. coelicolor* A3(2) (A21)	Hodgson, 1982 Hindle and Smith, 1994
Mannose	Mannose	Glucose	*S. alboniger* (A1B)	Surowitz and Pfister, 1985a
	Mannose	?	*S. violaceoruber* (A21)	Sabater *et al.*, 1972a

Table 5 *cont.*

Enzyme	Inducer	Repressor	Strain	Reference
Sucrose	Sucrose	Glucose	*S. alboniger* (A1B)	Surowitz and Pfister, 1985a
Xylose	Xylose	Glucose	*S. albus* (A16)	Sanchez and Smiley, 1975
	Xylose	Glycerol Glucose	*S. chrysomallus* (A1B)	
	Xylose	Glycerol Glucose	*S. olivochromogenes* (A19)	Roth *et al.*, 1987 Hanel *et al.*, 1987
	Xylose	Fructose Glucose	*S. phaeochromogenes* (A40)	
	Xylose, xylan	?	*S. flavogriseus* (A1C)	Chen and Anderson, 1979 Chen *et al.*, 1979 Ishaque and Kluepfel, 1981
	Xylose	?	*S. lividans* (A21)	Marcel *et al.*, 1987
	Xylose	?	*S. olivaceus* (A1C)	Parker, 1978
	Xylose	Glucose	*S. phaeochromogenes* (A40)	Sanchez and Quinto, 1975
	Xylose	Glucose	*S. rubiginosus* (A12)	Wong *et al.*, 1991
	Xylose	?	*S. violaceoniger* (A32)	Marcel *et al.*, 1987
	Xylose, xylan	?	*Streptomyces* sp.	Park and Toma, 1974

The promoters and terminators of the *S. coelicolor* A3(2) (A21) region have been mapped. There were two glycerol-inducible glucose-repressible promoters, gyl_{P1} and gyl_{P2}, that were responsible for transcription of the *gylCABX* operon; and a glycerol-inducible but poorly glucose-repressible promoter, $gylR_P$ for the *gylR* gene. All three promoters resemble the *E. coli* general consensus-like promoters identified previously in *S. coelicolor* A3(2). A series of related sequences, exhibiting dyad symmetry, were found adjacent to the gyl_{P1} and gyl_{P2} and $gylR_P$ promoters, and may be the sites of interaction of GylR. The terminators t′, t1 and t2 occur at the end of the coding regions of the *gylCA*, *gylB* and *gylX* cistrons. This organization is consistent with the requirement by the cell for glycerol-3-phosphate for reactions other than glycerol catabolism; e.g. in phospholipid synthesis and cell wall (techoic acid) synthesis. It might, therefore, be expected that glycerol transport (*gylC*) and glycerol kinase (*gylA*) would be required in the absence of glycerol-3-phosphate dehydrogenase (*gylB*). The nature of the regulation of termination is not understood (Smith and Chater, 1988a, b).

2.2.4.2. Galactose Catabolism. The galactose operon of *S. lividans* (A21) consists of three cistrons: *galTEK*, where *galT* encodes galactose-1-phosphate uridylyltransferase; *galE* encodes the UDPgalactose 4-epimerase; and *galK* encodes galactose kinase (Fornwald *et al.*, 1987; Adams *et al.*, 1988). The restriction site map is very similar to that of *S. coelicolor* A3(2) (A21) (Kendall *et al.*, 1987). There is no regulatory protein gene associated with the operon.

Again, as with the *gyl* operon, two promoters were found by S1 nuclease analysis. Promoter gal_{P1} is located 5′ of *galT* and was responsible for the generation of the *galTEK* polycistronic message. The second promoter, gal_{P2}, was situated between the *galT* and *galE* and produced a dicistronic *galEK* mRNA. The gal_{P1} promoter was galactose-inducible and glucose-repressible. The gal_{P2} promoter was expressed constitutively. This explains the observation that there is a constitutive low level of galactose kinase expression (Fornwald *et al.*, 1987). It has been suggested that the cell required a supply of galactose-1-phosphate and UDPgalactose for galactosyl lipid synthesis (Adams *et al.*, 1988) and perhaps cell wall synthesis. Hence *galE* and *galK* have an anabolic as well as a catabolic role. Adams *et al.* (1988) further reported that constitutive expression of *galK* is necessary for correct induction of gal_{P1} by galactose, implying that galactose-l-phosphate may be the true inducer of the operon, similar to glycerol-3-phosphate and the *gylCABX* operon. However, there has been a recent report that different forms of RNA polymerase with different sigma factors transcribe the two *gal* promoters (Brawner *et al.*, 1997). This is similar to the situation reported by Servin-Gonzalez *et al.* (1994) for the agarase gene of *S. coelicolor* A3(2) (A21). In the *gal* operon case, the physiological significance of the use of alternative sigma factors may be more obvious than in the *dagA* case because gal_{P1} and gal_{P2} are regulated differently.

The gene(s) responsible for regulation of the operon have not been identified. However, mutation of the proposed operator of gal_{P1} generated a promoter that was constitutively expressed and no longer subject to glucose repression (Mattern *et al.*, 1993). The simplest interpretation is that the mutated operator binds a repressor protein that also mediates glucose repression, similar to GylR. Ingram *et al.* (1995) reported the mutation of a gene, *crrA*, which led to constitutive, glucose-derepressed expression of gal_{P1} and the *gylCABX* operon.

2.2.4.3. Xylose Catabolism. Xylose metabolism in streptomycetes has received a lot of attention because the first enzyme of xylose catabolism, xylose isomerase, has the ability to interconvert glucose and fructose as well as xylose and xylulose; i.e. it is also a 'glucose isomerase'. The enzyme is used extensively in the food industry to produce high-fructose syrups from corn syrups. Because of this commercial interest, a number of groups have extensively characterized the enzyme from different species. Indeed, three-dimensional structures have been obtained for four streptomycete enzymes. Structures to a resolution of 3.0 Å have been obtained for the *S. rubiginosus* (A12), *S. olivochromogenes* (A19) and *S. violaceoniger* (A32) enzymes, and one of 1.9 Å for the *S. albus* (A16) enzyme (see Dauter *et al.*, 1989 for references).

The *S. violaceoniger* (A32) xylose isomerase gene (*xylA*) has been cloned (Marcel *et al.*, 1987) and sequenced (Drocourt *et al.*, 1988). Evidence was presented (Marcel *et al.*, 1987) that the other gene unique to xylose catabolism, xylulose kinase (*xylB*), was on the same piece of DNA as *xylA*. Whilst the two genes were coordinately regulated, there was no direct evidence of an operon structure. The *xylA* and *xylB* genes of *S. rubiginosus* (A12) have been cloned and sequenced and their regulation examined. The genes were independently transcribed and were induced about eight-fold by xylose. Glucose repressed this induction 3.8-fold for *xylA* and 1.3-fold for *xylB*. Mannitol had no effect on induction (Wong *et al.*, 1991).

Regulation of the production of xylose isomerase has been studied in a number of other streptomycetes (Table 5). In all cases the enzyme was inducible, either by xylose or by xylan, the xylose polymer. In a number of cases it has been reported that the enzyme is subject to glucose and/or glycerol repression.

Attempts have been made to isolate constitutive and/or glucose-derepressed xylose isomerase mutants. It was claimed that lyxose was a substrate for xylose isomerase, but not an inducer of it, and that mutants that could grow on lyxose as sole carbon source were constitutive for xylose catabolism genes (Sanchez and Quinto, 1975). However, Parker (1978) could not repeat these experiments and concluded that lyxose was not a substrate of xylose isomerase.

2.2.4.4. Fructose and Mannose Catabolism. Sabater *et al.* (1972b) identified a fructose-inducible, ATP-dependent, fructose kinase in *S. violaceoruber* (A21) that was unusual in its specificity for its substrate. This unusual specificity allowed it to be used in a specific assay for fructose (Sabater and Asensio, 1973b). The enzyme showed unusually complex kinetics which implied positive cooperativity induced by nucleotides and negative cooperativity induced by fructose (Sabater and Delafuente, 1975). These findings are perhaps surprising considering the catabolic role of fructose kinase. We might have expected that high ATP or low ADP would inhibit the enzyme whilst low ATP and high ADP would stimulate it; however, the opposite was the case. Inbar and Lapidot (1992) used ^{13}C NMR to study fructose metabolism in *S. parvulus* (A12). During rapid growth, fructose was rapidly transported into the cell and pools of mannitol, fructose and glucose-6-phosphate were formed rapidly. The accumulation of fructose and mannitol implied that fructose kinase was inactive in the strain; once glucose-6-phosphate decreased the enzyme appeared to be activated, implying the phosphosugar inhibited the enzyme. Examination of the growth conditions reveals an alternative explanation; i.e. fructose transport was constitutive but fructose catabolism has to be induced as in *S. coelicolor* A3(2) (A21) (Hodgson, 1982). It was not clear from the experiments that time had been allowed for fructose kinase induction.

Sabater *et al.* (1972a) identified a mannose-inducible, ATP-dependent, mannose kinase in *S. violaceoruber* (A21). This enzyme could also phosphorylate glucose, albeit with a lower affinity (4 mM) than for mannose (0.05mM). The inability of the enzyme to phosphorylate fructose was a unique feature.

2.3. Carbon Storage Compounds in Streptomycetes

The glucose homopolymer glycogen, the α-1,1-linked glucose disaccharide trehalose, polyhydroxybutyrate and neutral lipids such as triacylglycerols have been implicated as carbon storage molecules in streptomycetes. Studies indicate that these molecules have different roles in the cell, and that glycogen and trehalose are closely associated with morphological development (i.e. sporulation).

2.3.1. Trehalose

Every streptomycete so far examined contains trehalose in the vegetative mycelium (Elbein, 1967a, b, 1968; McBride and Ensign, 1987b). The pioneering work of Elbein showed that streptomycetes contain the disaccharide and that it was synthesized in a way different from the mechanism seen in insects and fungi. Streptomycetes used GDP-D-glucose as the donor to

glucose-6-phosphate in the formation of trehalose-6-phosphate. In insects and fungi the preferred donor is UDP-D-glucose. The K_m of the streptomycete enzyme for both of its substrates was reported to be 0.7 mM (Elbein, 1968). A specific trehalose-6-phosphate phosphatase has been identified in *S. hygroscopicus* (A32) (Hey-Ferguson *et al.*, 1973), as has trehalase, the enzyme responsible for cleaving the sugar to release glucose. Trehalase is the unique prerequisite for trehalose catabolism. The streptomycete trehalase could not cleave related disaccharides and had a K_m for its substrate of 18 mM (Hey and Elbein, 1968).

Hey-Ferguson *et al.* (1973) investigated the activity of the enzymes of trehalose anabolism (trehalose phosphate synthase and trehalose phosphate phosphatase) and catabolism (trehalase) during spore germination in *S. hygroscopicus* (A32). Trehalase activity increased after germination before outgrowth in a nutrient medium. There was a concomitant fall in trehalose. The phosphatase fell from a high activity in spores and levelled off only after outgrowth had begun. The trehalose phosphate synthase was low in the spores and increased with cell mass only when outgrowth had commenced. These observations are consistent with a role for trehalose as a carbon and energy store in streptomycete spores.

Inbar and Lapidot (1992) analysed the pathways of metabolism of fructose, glutamate and aspartate in *S. parvulus* (A12) using ^{13}C NMR. Trehalose was generated from fructose during the mid-log phase, and synthesis increased three-fold during the stationary phase and even further in the late stationary phase. Synthesis of trehalose also occurred, albeit to a lesser extent from glutamate and aspartate, but very little of this was via reverse glycolysis. Most appeared to be incorporated from pentose phosphate pathway intermediates.

Work on the location and kinetics of trehalose accumulation in *S. antibioticus* (A31) suggested a further role for the disaccharide in streptomycetes. Brana *et al.* (1986c) observed that accumulation occurred in the tips of the aerial mycelium and spores. They suggested that trehalose might have a role in osmoprotection. Catabolism of trehalose led to a concomitant loss of dehydration resistance. Addition of exogenous trehalose to such depleted cells led to the recovery of dehydration resistance, which might support the osmoprotection role (Martin *et al.*, 1986). Spores, which had been resuspended in distilled water, slowly catabolized trehalose without breaking dormancy. This might indicate a maintenance role in dormant spores. Therefore, there could be two roles for trehalose in streptomycetes: a dehydration resistance or osmoprotectant role in dormant spores; and as an energy and carbon source in germinating and, to a lesser extent, in wetted spores.

McBride and Ensign (1987a, b) confirmed and expanded the foregoing work to a number of other streptomycetes and actinomycetes. They demonstrated that the final concentration of trehalose in the spores was dependent on the culture conditions of the streptomycete. If the culture was grown on

glucose-limited media, the spores contained less trehalose. In contrast, limitation of the nitrogen or phosphate source stimulated trehalose accumulation. Prolonged incubation of spores, in conditions where germination did not occur, led to a slow decrease in trehalose content, whilst germination led to a rapid metabolism of trehalose. Addition of glucose to trehalose-depleted spores stimulated trehalose accumulation, even in conditions where protein and RNA synthesis was inhibited. Seventy-seven percent of all glucose taken up was stored as trehalose in untreated spores. These observations are consistent with the idea that trehalose has a role as a carbon and energy store within the spore.

McBride and Ensign were unable to find the germination-specific induction of trehalase activity that Hey-Ferguson *et al.* (1973) had seen in *S. hygroscopicus* (A32). Indeed, when they used the same strain as these authors, McBride and Ensign still could not repeat their observations. They consistently found a high activity of the enzyme in all spores, whether replete with, or deficient in, trehalose. The occurrence side by side of the catabolic enzyme and its substrate in streptomycete spores implied some kind of partitioning within the spore, or enzyme inactivation. Further examination (McBride and Ensign, 1990) revealed no evidence of either enzyme partitioning or modification. In addition, no evidence was found of trehalase inhibitors or activators. The authors were left with the conclusion that trehalase was inactive in dormant spores because of the dehydrated state of the spore. It was presumed that rehydration during spore germination was the trigger that activated the enzyme.

The osmotic strength of the medium had no effect on trehalose accumulation. This latter observation would imply that if trehalose was an osmoprotectant in *S. griseus* (A1B), accumulation was not osmotically regulated. Killham and Firestone (1984a, b) had reported that proline, glutamine and alanine were the osmoprotectants of *S. griseus* (A1B) and *S. californicus* (A9), and the accumulation of these amino acids was regulated osmotically.

McBride and Ensign (1987a) made the surprising observation that trehalose-replete spores germinated less efficiently and more slowly than trehalose-deficient spores. This might reflect the fact that dormancy is more difficult to break in trehalose-replete spores. As reported for *S. antibioticus* (A31), the heat and desiccation resistance of the spores increased with trehalose content. It should be noted, however, that even trehalose-depleted spores were more heat-resistant than mycelial fragments, and that heat-resistance to temperature levels lower than 55°C was independent of the trehalose content.

2.3.2. *Glycogen*

Hey-Ferguson *et al.* (1973) reported that the spores of *S. hygroscopicus* (A32) contained glycogen and that it was metabolized late in germination; essentially after the trehalose had been used up. However, no glycogen was reported in

mature *S. antibioticus* (A31) spores (Brana *et al.*, 1986c) or *S. venezuelae* (A6) (Ranade and Vining, 1993).

Brana *et al.* (1980) reported the accumulation and disappearance of polysaccharide granules during morphological development of *S. viridochromogenes* (A27). The first appearance of the granules was coincident with formation of sporulation septa in aerial hyphae. Once the sporulation septa were complete, the now enclosed immature spores filled with granules. About halfway through the spore maturation process the granules began to decrease in size and had disappeared by the time full maturation had been achieved. The kinetics of the process led the authors to suggest that the polysaccharide was a temporary carbon store which the organism used to hold the carbon and energy sources required later in the developmental process in an insoluble but readily mobilizable form. Later work (Brana *et al.*, 1982) revealed the polysaccharide to be glycogen. These authors also reported that a number of other streptomycetes contained glycogen as a carbon store.

Chemical and cytological analysis of the accumulation of glycogen in *S. antibioticus* (A31) confirmed the observations with *S. viridochromogenes* (A27). However, there is an additional glycogen accumulation step, phase I, in substrate mycelium at the onset of aerial mycelium formation. These reserves were catabolized as aerial mycelium development progressed. The second stage of glycogen accumulation, phase II, and degradation corresponded with the accumulation and disappearance of granules in immature spores. This led to the suggestion that phase-I accumulation, and the other products released during substrate mycelium lysis, were used as carbon and energy sources to support the growth of aerial hyphae (Mendez *et al.*, 1985; Brana *et al.*, 1986c). Altering the concentration of the nitrogen source supplied to the cells could modulate glycogen accumulation, but only when they had reached the correct developmental stage (Miguelez *et al.*, 1997).

Detailed electron microscopy confirmed an identical situation in *S. coelicolor* A3(2) (A21) to that of *S. antibioticus* (A31) (Plaskitt and Chater, 1995). Analysis of cloned glycogen synthesis (*glg*) genes revealed two separate gene clusters that encode isozymes for each step of the biosynthetic cluster. Inactivation of one cluster led to modification of one phase of glycogen accumulation. This has been shown to be true for *S. coelicolor* A3(2) (A21) (Bruton *et al.*, 1995; Martin *et al.*, 1997) and *S. aureofaciens* (A14) (Homerova and Kormanec, 1994; Homerova *et al.*, 1996).

2.3.3. Triacylglycerols

It was often assumed that bacteria do not accumulate neutral lipids, but Olukoshi and Packter (1994) demonstrated this assumption to be incorrect. It had often been noticed, during electron microscopy of thin sections, that

streptomycete hyphae appeared to contain vacuoles that may have been fat droplets (Plaskitt and Chater, 1995). Olukoshi and Packter (1994) demonstrated that *S. griseus* (A15), *S. albus* (A16), *S. coelicolor* A3(2) (A21) and *S. lividans* (A21) accumulated significant amounts of triacylglycerols, starting in the exponential phase and continuing into the late stationary phase. There was evidence of these lipids in the spores. Activity of the final enzyme of triacylglycerol synthesis, diacyglycerol acyltransferase, was found in *S. coelicolor* A3(2) (A21) and *S. lividans* (A21). The authors speculate that triacylglycerols provide the carbon units for antibiotic synthesis.

2.3.4. Polyhydroxybutyrate

There have been few reports of polyhydroxybutyrate formation in streptomycetes. This compound is formed by reduction and polymerization of acetoacetyl CoA and is a common storage compound in bacteria. Ranade and Vining (1993) reported small amounts (1 μg mg (dry weight)$^{-1}$) in *S. aureofaciens* (A14) and *S. antibioticus* (A31), and ten-fold less in *S. venezuelae* (A6). The results from the first two streptomycetes agreed with those of the first report from Kannan and Rehacek (1970b). The polymer was found only in the mycelium, not the spores, of *S. venezuelae* (A6) and was a minor storage compound. Glycogen and trehalose were the main storage compounds: trehalose in spores and glycogen in hyphae. Polyhydroxybutyrate could not be found in the hyphae of streptomycetes examined by Olukoshi and Packter (1994).

2.4. Carbon Catabolite Repression

2.4.1. Introduction

As noted above, catabolite repression of intracellular and extracellular catabolic enzymes and of carbohydrate transport systems have been reported in streptomycetes. Table 6 summarizes reports of repression by glucose, Table 7 summarizes reports of repression by carbohydrates other than glucose and Table 8 summarizes reports of regression by amino acids. It should be noted that the distinction used in these tables is rather arbitrary. In numerous instances catabolite repression of induction of an enzyme may, to different degrees, be exhibited by a number of catabolites. An example is the Class I cellobiase of *S. venezuelae* (A6) (Table 2). The induction of this enzyme can be repressed by glucose and, to a lesser extent, by amino acids and acetate.

As an object lesson, it might be relevant to consider what is probably a special case of indirect glucose repression. Redshaw *et al.* (1976) reported glucose repression of aerial mycelium of *S. alboniger* (A1B). They demonstrated that

Table 6 Systems Repressed by Glucose in Streptomycetes

Strain	System	Reference
S. alboniger (A1B)	Sucrose catabolism and transport Mannose catabolism and transport	Surowitz and Pfister, 1985a
S. albus (A16)	Xylose isomerase	Sanchez and Smiley, 1975
S. antibioticus (A31)	Fructose transport Galactose transport	Salas and Hardisson, 1981
S. coelicolor A3(2) (A21)	Agarase	Hodgson and Chater, 1981
	Arabinose catabolism and transport Fructose catabolism Galactose catabolism	Hodgson, 1982
	Glycerol catabolism and transport	Hodgson, 1982; Hindle and Smith, 1994
	Maltose transport	van Wezel *et al.*, 1997a
S. granaticolor (?)	Cellobiose transport β-1,4-glucosidase	Jiresova *et al.*, 1987
S. halstedii (A1C)	Cellulase	Fernandez-Abalos *et al.*, 1997
S. kanamyceticus (B42)	α-amylase	Flores *et al.*, 1993
S. lividans (A21)	Xylanase	Arhin *et al.*, 1994
	Galactose catabolism	Fornwald *et al.*, 1987
	Chitinase	Miyashita *et al.*, 1991
S. reticuli (?)	Cellulase	Walter and Schrempf, 1996a
S. rubiginosus (A12)	Xylose catabolism	Wong *et al.*, 1991
S. venezuelae (A6)	α-amylase	Virolle *et al.*, 1988
	β-1,4-glucosidase	Chatterjee and Vining, 1982b
	β-galactosidase	Chatterjee and Vining, 1982a
S. violaceus (A6)	β-galactosidase	Sanchez and Hardisson, 1980a

the repressive effect of glucose was via the accumulation of organic acids. As discussed above (Section 2.1.4), Surowitz and Pfister (1985a) demonstrated that the accumulation of organic acids was the result of glucose-induced uncoupling of glycolysis and the TCA cycle. This uncoupling could be reversed by adenine. I call this a special case because it appears that in this case 'glucose repression' is, in fact, glucose-induced metabolic poisoning. As discussed in section 2.1.4, glucose-induced organic acid accumulation is a common event in streptomycetes, depending on culture conditions, and a well-buffered medium needs to be used to ensure 'true' catabolite repression is involved.

Table 7 Systems repressed by carbohydrates other than glucose in streptomycetes.

Carbohydrate	Strain	System	Reference
Cellobiose	*S. coelicolor* A3(2) (A21)	Agarase Arabinose catabolism Fructose catabolism Galactose catabolism Glycerol catabolism	Hodgson, 1982
	S. lividans (A21)	Cellulase	Shareck *et al.*, 1987
	S. reticuli (?)	Cellulase	Walter and Schrempf, 1996a
	S. violaceus (A6)	β-galactosidase	Sanchez and Hardisson, 1980b
Fructose	*S. phaeochromogenes* (A40)	Xylose	Hanel *et al.*, 1987
	S. reticuli (?)	Cellulase	Walter and Schrempf, 1996a
Glycerol	*S. chrysomallus* (A1B)	Xylose	Hanel *et al.*, 1987
	S. kanamyceticus (B42)	α-amylase	Flores *et al.*, 1993
	S. lividans (A21)	Chitinase	Miyashita *et al.*, 1991
	S. olivochromogenes (A19)	Xylose	Hanel *et al.*, 1987
	S. reticuli (?)	Cellulase	Walter and Schrempf, 1996a
	Streptomyces sp. EC1	Cellulase β-1,4-glucosidase Xylanase β-1,4-xylosidase	Godden *et al.*, 1989
Mannitol	*S. limosus* (A1A)	α-amylase	Virolle and Bibb, 1988
	S. thermoviolaceus (C45)	α-amylase	Bahri and Ward, 1990

2.4.2. Amino Acid Repression of Carbohydrate Catabolism

Examples of amino acid repression of carbohydrate catabolism systems in streptomycetes are given in Table 8. Roth *et al.* (1985, 1986) and Grafe *et al.* (1986) investigated the repressive effect of amino acids on maltase production by amino acids in *S. hygroscopicus* (A32). They used a simple pH indicator plate test which was based on the observation that cells growing on maltose were acidogenic; cells growing on amino acids were alkalinogenic; and cells growing in the presence of maltose plus amino acids were alkalinogenic because they used the latter substrate preferentially. They reasoned that cells that had lost the amino acid repression system would be able to use maltose in

Table 8 Systems repressed by amino acids in streptomycetes.

Strain	System	Reference
S. clavuligerus (J71)	Glycerol transport	Minambres *et al.* 1992
S. hygroscopicus	α-amylase α-1,4-glucosidase	Grafe *et al.*, 1986 Roth *et al.*, 1986
S. parvulus (A12)	β-galactosidase	Foster and Katz, (1981)
S. venezuelae (A6)	α-1,4-glucosidase β-1,4-glucosidase	Chatterjee and Vining, 1981 Chatterjee and Vining, 1982b
S. violaceus (A6)	β-galactosidase	Sanchez and Hardisson, 1980a

the presence of amino acids and hence reduce the level of alkalinogenesis. Following mutagenesis and growth in a fermenter under a specifically designed selection regime, which involved switching the carbon and energy source from maltose to amino acids and back again over a number of cycles, they isolated a number of derepressed mutants. One of these mutants (M36) possessed a constitutive α-amylase and a constitutive maltase that were not subject to amino acid repression. A second mutant (M39) had lost amino acid repression of both enzymes but they remained inducible. The nature of the genetic regulatory network implied by these results – namely, a global activator of α-amylase and maltase and amino acid repression of the activator – has yet to be confirmed.

It should be noted that amino acid repression may have nothing to do with nitrogen regulation. Rather, the carbon skeletons left after deamination of the amino acids (i.e. organic acids) may be the 'true' repressor(s). Chatterjee and Vining (1981) observed that acetate and citrate were good repressors of the *S. venezuelae* (A6) maltase, and Kominek (1972) reported citrate 'repression' of glucose catabolism in *S. niveus* (A1B).

2.4.3. The Mechanism of Glucose Repression in the Enteric Bacteria

What do we know about carbon catabolite repression in streptomycetes? The short answer is 'not a lot', although there have been a number of important discoveries in the last few years. Glucose repression in streptomycetes shows very little similarity to glucose repression in the enteric bacteria (Postma *et al.*, 1993). The basis of the enteric system is the intracellular concentration of cyclic 3′,5′-AMP (cAMP). The DNA binding cAMP receptor protein (CRP) is

the key regulatory protein of the regulon. A number of catabolic operons (e.g. lactose, galactose, arabinose and histidine) are dependent on the presence of the cAMP–CRP complex for expression of genes at levels required for catabolism of their substrate. If intracellular concentration of cAMP is high, the cAMP–CRP complex forms and binds to DNA, leading to the binding of RNA polymerase holoenzyme by specific protein–protein interactions; provided, of course, there are no repressors to interfere in this interaction. The RNA polymerase can now initiate transcription of the operon, provided the appropriate inducers or anti-repressors are present.

The intracellular concentration of cAMP is controlled by: synthesis from ATP by adenyl cyclase; destruction by cAMP phosphodiesterase; and export out of the cell. It has been suggested that export is more important than destruction in its effect on cAMP concentration, as some 98% of cAMP present in a culture of *E. coli* is actually in the medium (Ullmann and Danchin, 1983) and cAMP phosphodiesterase has a very low affinity for its substrate.

Synthesis of cAMP is controlled as part of the PEP:PTS. The PEP:PTS comprises a phosphoprotein cascade linked to a class of carbohydrate-specific intramembrane permeases. The high-energy phosphate of PEP is transferred to the common PTS enzyme I. This phosphate is then transferred to a histidine residue on the common HPr protein, then the PTS glucose enzyme II subunit A ($EIIA^{Glc}$), formerly enzyme III. If there is glucose present outside the cell, the phosphate group on $EIIA^{Glc}$ is transferred to peripheral membrane protein $EIIB^{Glc}$ and then to the sugar as it is transported into the cell via the integral membrane $EIIC^{Glc}$ to form glucose-6-phosphate. If there is no glucose present outside the cell, phosphorylated $EIIA^{Glc}$ and phosphorylated HPr accumulate, and phosphate groups present on phosphorylated HPr can be used to phosphorylate carbohydrates other than glucose as they are imported via their respective PTS enzyme II complexes (e.g. EII^{Man} – the mannose transporter).

Phosphorylated $EIIA^{Glc}$ has a regulatory role. It is proposed that it activates adenyl cyclase. Therefore, if glucose is not present outside the cell, phosphorylated $EIIA^{Glc}$ accumulates which activates adenyl cyclase. This leads to an increase in intracellular cAMP which, in turn, can lead to the activation of genes involved in the catabolism of alternatives to glucose, provided they are present in the medium. If glucose is present in the medium, phosphorylated $EIIA^{Glc}$ is kept low because the phosphate group is being actively transferred to the glucose. Therefore, adenyl cyclase is not activated and so intracellular cAMP concentration is low.

Unphosphorylated $EIIA^{Glc}$ has an important regulatory role in inducer exclusion. It inhibits the class-I transport systems, which are responsible for the import of carbohydrates independently of the PTS system; e.g. lactose, maltose and mannitol, and glycerol kinase. If there is no glycerol kinase, glycerol is not removed from the intracellular pool and so the glycerol permease will stop importing glycerol as the concentration inside and outside equilibrates.

Unphosphorylated $EIIA^{Glc}$ is present during active transport of glucose, and so the class-I carbohydrates will not be transported when glucose is being transported.

It should be stressed that this model does not explain all the observed phenomena concerning carbohydrate repression and inducer exclusion in *E. coli*. The role of cAMP in regulation in bacteria has been reviewed critically by Ullmann and Danchin (1983).

2.4.4. The Mechanism of Glucose Repression in Low GC Ratio Gram-positive Bacteria

A similar PEP:PTS relay has been identified in *Bacillus* and *Staphylococcus* species but there is no involvement of adenyl cyclase. Again PEP transfers phosphate to a common enzyme I. This phosphate in turn is transferred to a histidine residue on HPr from where it can be transferred to $EIIA^{Glc}$, then $EIIB^{Glc}$, then to glucose as it is imported via $EIIC^{Glc}$. If there is no glucose, histidinyl phosphorylated HPr can stimulate the activities of glycerol kinase and regulators of catabolism operons such as LicT; thus carbohydrates other than glucose can be used. If glucose is present, a protein kinase, PtsK, phosphorylates HPr and the HPr-like protein Crh at serine residues. This serinyl phosphorylated HPr and serinyl phosphorylated Crh can act as co-repressors with CcpA. CcpA and its co-repressor bind to *cre* sequences within promoter regions of genes involved in carbohydrate and amino acid catabolism; e.g. xylose and histidine, respectively. The serinyl phosphorylated HPr also interacts with sugar permeases and causes expulsion of the sugar; i.e. inducer expulsion. Thus if glucose is present the alternative catabolism systems are repressed and inducers are excluded from the cell (reviewed in Stuelke and Hillen, 1999; and Poolman, 1999).

2.4.5. Carbon Carbohydrate Repression in Streptomycetes

2.4.5.1. Diauxic Growth and Carbohydrate Repression. Diauxic lag is a phenomenon characteristic of glucose repression and inducer exclusion in the enteric bacteria. When a cell is presented with growth-limiting glucose and excess glucose-repressible catabolite, the cell metabolizes the glucose alone first. Once glucose is exhausted, the cell ceases growing until the enzymes necessary for the catabolism of the second catabolite have been induced. This period of growth cessation is referred to as the 'diauxic lag'.

A number of groups have sought to demonstrate diauxic lag in streptomycetes cultures supplied with growth-limiting repressing catabolite and excess repressible catabolite. Chatterjee and Vining (1982a) reported a diauxic lag when *S. venezuelae* (A6) was incubated in a medium initially containing

0.6% glucose and 1.4% lactose. Analysis of residual sugar in the medium revealed the complete catabolism of the glucose, the exhaustion of which correlated with the initiation of the lag in cell growth. The lag ended upon initiation of lactose catabolism. When the same strain was incubated in the presence of 0.6% glucose and 1.4% cellobiose, the glucose was not exhausted before cellobiose catabolism commenced, and no diauxic lag was seen (Chatterjee and Vining, 1982b).

Hodgson (1982) could not find a diauxic lag phase when *S. coelicolor* A3(2) (A21) was grown in medium containing growth-limiting glucose (0.56 mM) and excess alternative (glucose repressible) carbon and energy source. Diauxic growth could be seen, however, as the cells grew with the characteristic doubling time (1.9 h) of glucose first and then slowed down to the characteristic doubling time seen for growth on the alternative carbon source. The point of changeover from the first phase to the second occurred at precisely the point where growth ceased in cultures initially supplied with 0.56 mM of glucose alone. Hanel *et al.* (1987) were also unable to find a diauxic lag phase when the growth of *S. chrysomallus* (A1B) on glucose and xylose was examined. Glucose and xylose were co-catabolized, although glucose was preferentially used.

Since glucose transport systems in streptomycetes have low affinity for their substrate – K_m values of 0.12 mM to 6.1 mM (Table 4) – glucose limitation could occur even though glucose was still present in the medium. Taking this into consideration with the observations that comparative growth rates of streptomycetes are slower than those of enteric bacteria, whilst the time taken to activate, transcribe and translate genes are about the same, it is perhaps not surprising that diauxic lags are rarely seen in streptomycetes. It should also be noted that the PEP:PTS-dependent mechanism of inducer exclusion makes a significant contribution to delay of induction of the catabolism genes, and thus to the creation of diauxic lag.

2.4.5.2. Inducer exclusion. As mentioned above (section 2.2.3.) a number of constitutive carbohydrate transport systems of streptomycetes are inhibited by glucose (Table 4). This raises the possibility that, where the carbohydrate catabolism enzymes are inducible, the repressing carbohydrate could exert its repressing effect by excluding the inducer. Whilst inducer exclusion may have a role in catabolite repression, it cannot be the sole mechanism if the results of Hodgson (1982) in *S. coelicolor* A3(2) (A21) have a more universal applicability. Mutants were isolated that had lost the mechanism of glucose repression (section 2.4.5.4); however, these mutants still retained the ability for glucose to inhibit transport of inducers (in this case fructose and galactose) into the cell. Thus, when these mutants were cultured in the presence of glucose and the inducer, enough inducer could get into the cell to fully induce the catabolic enzymes. Servin-Gonzalez *et al.* (1994) also concluded inducer exclusion was

not the basis of glucose repression of agarase induction in *S. coelicolor* A3(2) (A21).

2.4.5.3. Is Cyclic 3′,5′-AMP Involved in the Control of Glucose Repression of Streptomycetes? It has been shown to occur in a number of streptomycetes (Table 9). The concentration of the nucleotide varies, however, from 4 to 400 pmol (mg protein)$^{-1}$ (Dobrova *et al.*, 1984). Only a minority (about 10%) of the cAMP present in a culture is intracellular (Dobrova *et al.*, 1984; Lishnevskaya *et al.*, 1986), the rest being present in the culture medium. The presence of cyclic 3′,5′-GMP has also been reported in *S. hygroscopicus* (A32) (Gersch *et al.*, 1978).

Attempts to implicate cAMP as the regulatory molecule of carbon catabolite repression of both primary and secondary metabolism have failed to provide unambiguous results in all the species examined (Table 9). In a number of cases (Gersch, 1980; Surowitz and Pfister, 1985b; Lishnevskaya *et al.*, 1986) it was found that, in contrast to what is seen in *E. coli*, intracellular cAMP concentration was greatest in media containing glucose where growth rate was highest and lowest in media containing either no carbon and energy source or one that could not be catabolized. In contrast, Dobrova *et al.* (1984) reported that total and intracellular concentration of cAMP in *S. granaticolor* (?) was lowest during active growth and increased only after growth had ceased. However, they reported that cAMP synthesis was possible only in the presence of a carbon and energy source, and confirmed that adenyl cyclase was most active in cells with a utilizable carbon and energy source. In this case it appeared that cAMP had the kinetics of a secondary metabolite.

In summary, high growth rate correlates with high intracellular cAMP concentration; and low growth rate correlates with low intracellular cAMP level. This situation is reminiscent of that in the yeast *Saccharomyces cerevisiae* (Postma, 1986).

Additional correlations of growth events with cyclic nucleotide metabolism in streptomycetes have been reported. Gersch *et al.* (1979a) and Amini (1994) reported that cAMP synthesis in *S. hygroscopicus* (A32) and *S. coelicolor* A3(2) (A21), respectively, was correlated with spore germination, although addition of exogenous cAMP inhibited germination of the former streptomycete. Ensign (1982) reported that, during germination of *S. viridochromogenes* (A27) spores, the high cAMP concentration within the spore was reduced by excretion into the medium. Gersch also reported (1979) that exogenous cAMP was a stimulator of protein, RNA and DNA synthesis in *S. hygroscopicus* (A32) vegetative mycelia. The concentrations of exogenous cAMP (5–10 mM) required to inhibit germination and stimulate vegetative growth were the same (Gersch and Strunk, 1980). Ring *et al.* (1977a, c) reported that exogenously supplied cAMP could mimic autogenous stimulation of amino acid transport systems by their substrates. This stimulation occurred apparently at the level of transcription.

Table 9 Reports of cyclic 3′5′-AMP and its effects in streptomycetes.

Strain	Examined Role	Reference
S. alboniger (A1B)	No role in aerial mycelium formation. Glucose stimulated cAMP production	Surowitz and Pfister, 1985b
S. antibioticus (A31)	No role in glucose repression of galactose catabolism or regulation of actinomycin production	Chatterjee and Vining, 1982c
	Correlation of cAMP production with growth rate. No role in regulation of oleandomycin production.	Lishnevskaya *et al.*, 1986
S. coelicolor A3(2) (A21)	No role in glucose repression of arabinose, fructose, galactose and glycerol catabolism	Hodgson, 1980
	Greatest production of cAMP in early exponential phase following germination. No correlation of cAMP production with carbon, nitrogen or phosphate source. Correlation of cAMP production with growth rate.	Amini, 1994
	Greatest production of cAMP in early exponential phase following germination, and fresh peaks associated with aerial mycelium formation and actinorhodin production. Mutant in cAMP production: (1) Germ tube formation severely delayed or abolished; (2) Could not neutralize auto-acidified medium, therefore aerial mycelium formation blocked; (3) Actinorhodin production blocked	Susstrunk *et al.*, 1998
S. cyanogenus (?)	No role in carbon or nitrogen catabolite repression of purine catabolism	Watanabe *et al.*, 1973, 1976a Ohe and Watanabe, 1978
S. fradiae (G68)	Chloroquine increased DNA content and cAMP and tylosin production. A mutant was found with increased DNA content and cAMP and tylosin production.	Colombo *et al.*, 1982

Table 9 cont.

Strain	Examined Role	Reference
	Exogenous cAMP stimulated tylosin production in nutritionally poor medium and sporulation in nutritionally rich medium.	Tata and Menawat, 1994
S. granaticolor (?)	No role in catabolite repression. Synthesis correlated with presence of a carbon and energy source.	Dobrova *et al.*, 1984
S. griseus (A15)	No role in regulation of candicidin synthesis. No role in regulation of streptomycin synthesis. No role in phosphate regulation of streptomycin synthesis.	Martin and Demain, 1977 Ragan and Vining, 1978 Terry and Springham, 1981
S. hydrogenans (A5)	Exogenous cAMP stimulated amino acid permiase production	Ring *et al.*, 1977a, c
S. hygroscopicus (A32)	Negative correlation of cAMP production and turimycin production	Gersch *et al.*, 1978
	Exogenous cAMP reversed phosphate repression of turimycin production	Gersch *et al.*, 1979b
	Exogenous cAMP inhibits germination.	Gersch *et al.*, 1979a Gersch and Strunk, 1980
	Exogenous cAMP stimulates growth.	Gersch, 1979 Gersch and Strunk, 1980
	Membrane depolarisation stimulates cAMP formation.	Gersch and Romer, 1982
S. kanamyceticus (B42)	Exogenous cAMP relieved glucose repression of *N*-acetylkanamycin aminohydrolase	Satoh *et al.*, 1976
S. venezuelae (A6)	No role in glucose repression of β-galactosidase or regulation of chloramphenicol production.	Chatterjee and Vining, 1982a
	No role in glucose repression of β-glucosidase	Chatterjee and Vining, 1982b

Intriguingly, intracellular cAMP levels, cAMP transport, cAMP synthesis and cAMP destruction oscillated throughout the cell cycle in a number of actinomycetes (Lefebvre *et al.*, 1978a, b; Quennedey *et al.*, 1978). The actinomycetes studied, *Arthrobacter globiformis*, *Nocardia restricta* and *Rhodococcus* sp., were synchronized by transferring stationary phase cultures to fresh media and synchronization monitored by thymidine incorporation into DNA. In each case the concentration of cAMP rose to a maximum (at least three times that of the minimum) once during each cell cycle. To the author's knowledge no explanation for this phenomenon has been reported, nor has the same observation been made in any streptomycete.

It is perhaps of interest to note the cell cycle-dependent fluctuation of trehalase, a cAMP-dependent protein kinase activated enzyme in *Saccharomyces cerevisiae* (van Doorn *et al.*, 1988). This report indicated that the absence of trehalase activity was due to the absence of the activity of the cAMP-dependent protein kinase. It is not yet clear whether the cell cycle fluctuation was the result of a cyclic appearance and disappearance of cAMP, or of the protein kinase.

The most unequivocal studies of cAMP in a streptomycete have been that of Susstrunk *et al.* (1998) following the isolation of a mutant of *S. coelicolor* A3(2) that could not produce cAMP. This *cya* mutant was isolated by disrupted gene replacement following cloning of the adenyl cyclase gene from the streptomycete (Danchin *et al.*, 1993). In the wild type, the peak of cAMP production was at germination and only 15% of the spores of the mutant formed germ tubes, and formation was severely delayed. There were also peaks of cAMP production in the wild type at the onset of aerial mycelium formation and initiation of antibiotic production. Colonies of the *cya* mutant failed to form aerial mycelia unless the medium was pH-buffered or cAMP was added exogenously. Studies with wild type and mutant revealed that both produced acid during substrate mycelium formation, but that wild type was able to neutralize the acid, presumably by consumption, and initiate aerial mycelium formation, whereas the mutant could not neutralize the acid. This is similar to the situation in *S. alboniger* (A1B) (Surowitz and Pfister, 1985a) in which uncoupling of glycolysis and the TCA cycle led to acid secretion and inhibition of aerial mycelium formation (sections 2.1.4 and 2.4.1). Supplementation of the *cya* mutant with cAMP also allowed the resumption of antibiotic formation.

The importance of medium acidification in the cAMP mutant raised some interesting questions. A number of mutants of *S. coelicolor* A3(2) (A21), *bldA*, *bldC*, and *bldD*, were isolated that could not produce aerial mycelium in the presence of glucose but could in its absence when growing on 'non-repressing' carbohydrates such as mannitol (Merrick, 1976). It was suggested that, in these mutants, development had become glucose-repressible. Susstrunk *et al.*, (1998) demonstrated that *bldA*, *bldB*, *bldC*, *bldD* and *bldG* mutants irreversibly

acidified the medium when growing on glucose. Addition of cAMP or pH-buffering the medium did not suppress the Bld$^-$ phenotype. It would be interesting to see whether the mannitol-suppressible *bld* mutants produced acid on 'non-repressing' carbohydrate catabolites. Again the observation of acid formation and 'glucose repression' are seen to be linked in streptomycetes. We conclude that cAMP has nothing to do with the mechanism of carbon catabolite repression in streptomycetes.

2.4.5.4. A Role for Glucose Kinase in Glucose Repression in Streptomycetes. Hodgson (1982) studied glucose repression of primary metabolism in *S. coelicolor* A3(2) (A21). Having shown that a number of carbohydrate transport and catabolism systems were glucose-repressed, glucose-derepressed mutants were selected on media containing arabinose, a glucose-repressed catabolite, and excess of the non-catabolizable glucose-analogue, 2-deoxyglucose. The idea was that as the 2-deoxyglucose could not be catabolized, yet it repressed the use of arabinose, mutants would be selected that had lost the glucose repression system. Such mutants were isolated and appeared to have lost the ability to grow on glucose because of the loss of glucose kinase. Initially the evidence for loss of the enzyme was based on sugar catabolism patterns and glucose uptake studies. Biochemical studies confirmed this conclusion (Seno and Chater, 1983).

Angell *et al.* (1992) reported the DNA sequence analysis of an open reading frame (ORF3) that complemented the glucose kinase mutants. The predicted protein showed homology with a xylose catabolism operon (repressor protein, XylR, from *Bacillus subtilis* and *Lactobacillus pentosus*) and the *N*-acetylglucosamine catabolism operon (repressor protein, NagC, from *E. coli*). This raised the possibility that the ORF3 encoded a regulator of glucose kinase and not the structural gene itself. This possibility was rejected since the ORF3 lacked the putative DNA binding domains present on the repressor proteins, and expression of ORF3 alone in *E. coli* led to a new glucose kinase activity with the same molecular weight as the *S. coelicolor* A3(2) activity. ORF3 was transcribed from two promoters; the more powerful transcribed ORF3 alone and was unaffected by growth on galactose rather than glucose. Transcription of ORF3 decreased as the cells entered the stationary phase.

Angell *et al.* (1994) reported that glucose kinase had a regulatory role in addition to its glucose phosphorylation function. Isolation of suppressers of the glucose non-utilization phenotype of the *glkA* deletion mutants yielded unstable strains that could grow on glucose, had glucose kinase activity and yet failed to glucose-repress agar utilization. The authors speculated that there could have been activation of a silent glucose kinase gene in the suppresser strains. Recently the genome-sequencing project has yielded a gene with extensive similarity to the *glkA* gene (AL109950). The fact that a strain with wild-type levels of glucose kinase activity failed to exhibit glucose repression implies that glucose phosphorylation activity was not important in glucose

repression. This was proven when the glucose kinase gene of *Zymomonas mobilis* was expressed in the *glkA* deletion mutant and shown to produce substantial glucose kinase activity, yet the strain failed to exhibit glucose repression of agarase. Clearly glucose flux through the glycolytic pathways or into the cell cannot be important in the control of glucose repression in streptomycetes. The regulatory role for glucose kinase in glucose repression in *S. coelicolor* A3(2) was confirmed by Kwakman and Postma (1994), and they also demonstrated that ten-fold over-expression of the *glkA* gene led to loss of glucose repression, implying that the repressive signal was titratable.

Hodgson (1982) was fortuitous in the choice of 2-deoxyglucose for selection of glucose-derepressed mutants. The product of the reaction of the analogue with the kinase, 2-deoxyglucose-6-phosphate, like all other phosphorylated sugars, is toxic to the cell because of its ability to react destructively with proteins and DNA (Lee and Cerami, 1987). Therefore the only cells that could survive either could not import it or could not phosphorylate it; i.e. be mutant in the glucose kinase. If *S. coelicolor* A3(2) (A21) has more than one glucose transport system, like its close relative *S. lividans* (A21) (Table 4), transport mutants would not be a class of analogue-resistant mutants. A further glucose-derepressed mutant of *S. coelicolor* A3(2) was isolated by screening for loss of glucose-repression of the extracellular enzyme agarase (Hodgson, 1982). Again this mutant proved to have lost glucose kinase activity.

There are some anomalies in the glucose kinase story. When the chitinase gene *chi63* cloned from *S. plicatus* (A12) was introduced into *S. coelicolor* A3(2) (A21), it was found to be chitin-inducible and glucose-repressible, but glucose repressibility was still seen in a *glkA* mutant (Ingram and Westpheling, 1995). This surprising result was made more surprising in that the native chitinase in the close relative, *S. lividans* (A21), was found to be dependent on *glkA* for glucose repression (Saito *et al.*, 1998). The *chi63* case may be anomalous in that the gene is being expressed in a heterologous host. There remains the possibility that inducer exclusion may play a role. We do not know what the true inducer is; presumably it is a breakdown product of chitin, and that inducer may not be transported into the cell in *S. coelicolor* A3(2) (A21) in the presence of glucose. There was a similar situation when the *S. reticuli* (?) α-amylase was expressed in *S. coelicolor* A3(2); the gene was still inducible and glucose-repressible in a *glkA* mutant (Walter and Schrempf, 1996a, b).

Another apparent anomaly is the observation that the mannitol-repressed α-amylase gene of *S. limosus* (A1A), when transferred to *S. coelicolor* A3(2) (A21), retained maltose induction but became subject to *glkA*-dependent glucose repression (Virolle and Bibb, 1988). This implies that the regulatory role of the *glkA* glucose kinase can substitute for the regulatory activity (mannitol kinase?) in *S. limosus* (A1A). Flores *et al.* (1993) reported that glucose kinase did not appear to be involved in glucose or glycerol repression of α-amylase in *S. kanamyceticus* (B42). McMahon *et al.* (1997) provided evidence

that growth rate had an important role in catabolite repression of the α-amylase of an unidentified streptomycete, *Streptomyces* sp. IMD 2679.

A new gene, *sblA*, was identified in a search of transposon mutants that had lost glucose repression of the *S. limosus* (A1A) α-amylase gene, *aml*, in *S. lividans* (A21). This gene encodes a protein with homology to a phosphatase whose substrates are small molecules. Loss of the gene led to partial loss of glucose repression of the *aml* gene, but no other glucose-repressed genes were tested. The mutant had reduced growth and growth rate on glucose and glycerol (Gagnat *et al.*, 1999). It is tempting to speculate that the phosphorylase action may have some role in control of sugar phosphate levels, and hence glucose repression. However, it was not reported if any sugar phosphates were a substrate and the loss of the phosphatase might have been expected to increase glucose repression rather than relieve it.

The concomitant loss of a global glucose repression system and glucose kinase activity is very reminiscent of the situation in *Saccharomyces cerevisiae*, where the hexokinase PII isozyme has been strongly implicated in control of glucose repression (Carlson, 1987). The nature of the role of this enzyme in glucose repression is not yet clear.

2.4.5.5. The Involvement of Carbohydrate Catabolism Gene Regulators in Glucose Repression. The observation in *S. coelicolor* A3(2) (A21) that the loss of the repressor GylR of the glycerol catabolic operon *gylCABX* led to constitutive expression of the operon and loss of glucose repression clearly implicates the repressor in the mediation of the glucose repression mechanism (Hindle and Smith, 1994). A similar observation was observed with MalR, the repressor of the *malEFG* operon (van Wezel *et al.*, 1997a). The product of the *malR* gene in *S. lividans* (A21), *reg1*, was also found to control both induction by the appropriate inducer and glucose repression of chitinase and α-amylase (Nguyen *et al.*, 1997a). There was also evidence that the *S. limosus* (A1A) α-amylase, which was mannitol-repressed in *S. limosus* but glucose-repressed in *S. lividans*, was under the control of Reg1/MalR. If glucose repression is mediated via MalR, we finally have a mechanistic explanation for the change in repressor between the two streptomycetes. In agreement with the role of these repressors in glucose repression comes the observation that mutation of the proposed operator of the galactose-inducible, glucose-repressible *gal* promoter (Mattern *et al.*, 1993) and operator of the chitin-inducible, glucose-repressible *chi63* promoter (Ni and Westpheling, 1997) led to constitutive, glucose-resistant expression of both the promoters. The proposed repressor of the *S. coelicolor* A3(2) (A21) agarase gene, *dagA*, appears to be an exception to the rule in that when the gene became constitutive it still retained glucose-repressibility (Servin-Gonzalez *et al.*, 1994).

It is tempting to speculate that the mechanism of glucose repression in streptomycetes involves an interaction between the *glkA* glucose kinase and the

different catabolism operon repressors. Whether the interaction between the repressors and the kinase is direct or indirect has yet to be determined.

2.4.5.6. Pleiotropic Mutations and Glucose Repression. Pope *et al.* (1996) reported that *S. coelicolor* A3(2) (A21) bald mutants, as well as having lost the ability to form an aerial mycelium and antibiotics, had also lost the ability to regulate primary catabolism. Glucose repression was lost and in some mutants galactose catabolism no longer depended on galactose induction. A related observation had been made previously in this strain in that all the bald mutants then available (*bldA*, *bldB*, *bldC* and *bldD*) were no longer sensitive to 2-deoxyglucose when supplied with arabinose as sole carbon source (Hodgson, 1980). We now know from the work of Susstrunk *et al.* (1998) that bald mutants are deregulated in acid production (see section 2.4.5.3). It is difficult to assess the significance of any particular aspect of the complex phenotype of these bald mutants. It is known that *bldA* encodes a rare leucine codon, tRNA (Leskiw *et al.*, 1991), and *bldB* and *bldD* each encodes a small protein with a potential DNA-binding site that is subject to autogenous regulation (Elliot *et al.*, 1998; Pope *et al.*, 1998). Another pleiotropic mutation that had deregulated a number of carbohydrate catabolism genes was the *ccrA1* mutation (Ingram *et al.*, 1995). The gene(s) affected were not identified so we cannot see where they fit into the glucose repression story.

2.5. Carbon Metabolism: General Conclusions

Control of degradation of carbon catabolites in streptomycetes is similar to that seen in other bacteria (Table 10; Fig. 1). There are many examples of induction of the catabolic pathway by the primary catabolite (Table 10). However, it is difficult to see how induction by polymeric substrates can occur if they are too large to be imported into the cell. Presumably a small, soluble breakdown molecule is the messenger that informs the cell of a potential food source present outside the cell. In some cases the substrate may be very complex (e.g. ligno-cellulose and chitin), in which case an entire complex of extracellular enzymes has to be induced. It may be necessary for this complex to include enzymes that cannot release a catabolizable product but which allow other enzymes access to their substrate that will release catabolites. In cases such as this, inducers of enzyme complexes may be found that are not catabolites and have no structural relationship to the substrate or product of many of the enzymes released as part of the complex.

Studies of galactose, glycerol and maltose catabolism reveal that regulation of catabolism of simple carbohydrates appears to be similar to that seen in other bacteria; i.e. there is a repressor protein, which binds to operators in the promoter regions of the catabolic operon in the absence of the substrate.

Table 10 Levels of Induction of Inducible Catabolic Systems in Streptomycetes

System	Induction level	Strain	Reference
α-amylase	40	*S. cellulosae* (A13)	
(α-1,4-endoglucanase)	21	*S. diastaticus* (A19)	
	13.1	*S. griseus* (A15)	Simpson and McCoy, 1953
	20	*S. microflavus* (A23)	
	171	'Thermophile'	
	18.3	*S. hygroscopicus* (A32)	Grafe *et al.*, 1986
	20	*S. kanamyceticus* (B42)	Flores *et al.*, 1993
	9.7	*S. limosus* (A1A)	Virolle and Bibb, 1988
	>20	*S. venezuelae* (A6)	Virolle *et al.*, 1988
	50	*Streptomyces* sp. IMP2627	McMahon, *et al.*, 1997
α-glucanase	>248	*S. chartreusis* (A18)	Inoue *et al.*, 1975
(α-1,3-endoglucanase)	>184	*S. werraensis* (A12)	Takehara *et al.*, 1981
	>13.1	*Streptomyces* sp. KI-18	Imai *et al.*, 1977a
Cellulase	53.3	*Streptomyces* sp. EC1	Godden *et al.*, 1989
(β-1,4-endoglucanase)	108	*Streptomyces* sp. QM-B814	Perez-Pons *et al.*, 1994
CMCase	238	*Streptomyces* sp. QM-B814	Perez-Pons *et al.*, 1994
Laminarinase	46	Thermophilic streptomycete	Lilley and Bull, 1974
(β-1,3-glucanase)	252	*Streptomyces* sp. QM-B814	Perez-Pons *et al.*, 1994
Xylanase	27.4	*S. flavogriseus* (A1C)	Kluepfel and Ishaque, 1982
(β-1,4-endoxylanase)	13.6	*S. lividans* (A21)	Vats-Mehta *et al.*, 1990
	21	*Streptomyces* sp.	Park and Toma, 1974
	75.4	*Streptomyces* sp. EC1	Godden *et al.*, 1989
Mannanse (β-1,4-endomannase)	>45	*S. olivochromogenes* (A19)	Ratto and Poutanen1988.

Table 10 cont.

System	Induction level	Strain	Reference
Chitanase	66	*S. lividans* (A21)	Miyashita *et al.*, 1991
(β-1,4-endoacetylglutaminase)	33	*S. plicatus* (A12) in *S. lividans* (A21)	Robbins *et al.*, 1992
Maltase	9.6	*S. hygroscopicus* (A32)	Roth *et al.*, 1985
(α-1,4-glucosidase)	43	*S. venezuelae* (A6)	Chatterjee and Vining, 1981
Cellobiase	500	*S. granaticolor* (?)	Jiresova *et al.*, 1987
(β-1,4-glucosidase)	14	*S. reticuli* (?)	Heupel *et al.*, 1993
	8.7	*S. venezuelae* (A6)	Chatterjee and Vining, 1982b
	455	*Streptomyces* sp. EC1	Godden *et al.*, 1989
	10.4	*Streptomyces* sp. QM-B814	Perez-Pons *et al.*, 1995
	6.5–8	*Streptomyces* sp. UM-2	Jaurin and Granstrom, 1989
	5.5–10.7	*Streptomyces* sp. UM-d	Jaurin and Granstrom, 1989
Xylosidase (β-1,4-xylosidase)	400	*Streptomyces* sp. EC1	Godden *et al.*, 1989
β-galactosidase	8.3	*S. griseus* (A15)	Dan and Szabo, 1973
(β-1,4-galactosidase)	3.1	*S. venezuelae* (A6)	Chatterjee and Vining, 1982a
	18.3	*S. violaceus* (A6)	Sanchez and Hardisson, 1980a
Cellobiose Transport	250	*S. granaticolor* (?)	Jiresova *et al.*, 1987
	>10	*S. reticuli* (?)	Schloesser and Schrempf, 1996
Fructose Transport	>33	*S. antibioticus* (A31)	Salas and Hardisson, 1981
	27	*S. coelicolor* A3(2) (A21)	Titgemeyer *et al.*, 1995
	3.2	*S. venezuelae* (A6)	Tang, 1977
Galactose Transport	>44	*S. antibioticus* (A31)	Salas and Hardisson, 1981

Table 10 cont.

System	Induction level	Strain	Reference
Fructose Kinase	>100	*S. violaceoruber* (A21)	Sabater *et al.*, 1972a
Galactose Catabolism	>12.8	*S. lividans* (A21)	Fornwald *et al.*, 1987
Glycerol Catabolism	35	*S. coelicolor* A3(2) (A21)	Seno and Chater, 1983
Mannose Kinase	>70	*S. violaceoruber* (A21)	Sabater *et al.*, 1972a
Xylose Catabolism	>100	*S. albus* (A16)	Sanchez and Smiley, 1975
	20	*S. chrysomallus* (A1B)	Roth *et al.*, 1987
	5.1	*S. flavogriseus* (A1C)	Chen *et al.*, 1979
	27.1	*S. flavogriseus* (A1C)	Ishaque and Kluepfel, 1981
	17.7	*S. olivaceus* (A1C)	Parker, 1978
	35	*S. phaeochromogenes* (A40)	Sanchez and Quinto, 1975

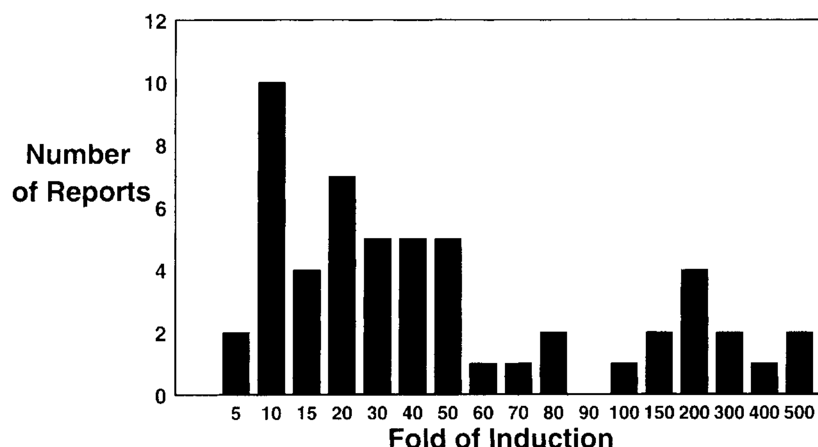


Figure 1 Induction of carbohydrate catabolism in streptomycetes (see Table 10 for data and references).

Another important common observation is that the streptomycete carbon catabolite repression mechanism closely involves the repressor proteins. Studies on MarR/Reg1 reveal at least one example of control of many disparate gene clusters in a coordinated fashion (i.e. regulon control).

An unusual feature of streptomycete physiology is the apparent low affinity of many of the carbohydrate transport systems (Table 4). In other bacteria, carbohydrate transport systems have affinities within the micromolar range. The K_m values of the streptomycete permeases vary from 1 mM to 47 mM. A number of streptomycete carbohydrate transport systems have been observed that have affinities more in line with other bacteria (Table 3); however, all of these systems are inducible by their substrates.

The fact that streptomycete sugar transport systems have low affinities for their substrate might lead one to suggest that they are not capable of transporting the carbohydrate against a concentration gradient; i.e. they are facilitators rather that active transport systems. However, Hodgson (1982) and Garcia-Dominguez *et al.* (1989) have reported that low-affinity transport can be inhibited by addition of uncoupling agents and inhibitors of respiration, indicating that they are active. The latter paper also demonstrated that glucose could be concentrated 20-fold from the medium.

The occurrence of the low-affinity streptomycete carbohydrate permeases may be a consequence of soil streptomycetes growing by the solubilizing of complex polysaccharides using extracellular enzymes. If the concentration of these polysaccharides is high, the local concentration of monomers and oligomers will be very high; i.e. in the mM range. As stated in the introduction,

complex polysaccharides are at high concentration in soil because soil is derived mainly from plant material and plants are carbon-rich but nitrogen-poor.

3. NITROGEN METABOLITE CATABOLISM

The provision of nitrogen sources for growth of streptomycetes is important when considering their commercial exploitation. Many streptomycete secondary metabolites are subject to 'nitrogen repression'. This term, which is borrowed from the study of control of primary metabolism, is liable to misinterpretation. In primary metabolism, nitrogen repression is a mechanism whereby a hierarchy of nitrogen sources is exploited sequentially; e.g. ammonium, glutamate and glutamine are preferred to nitrate or histidine. As a rule, the nitrogen sources used first are the easiest to assimilate; i.e. the ones requiring the minimal expenditure of energy and reducing power. Ammonium repression in enteric bacteria ensures that the systems of amino acid catabolism etc. are not activated if plentiful ammonium is present. The mechanisms are complex and involve gene expression regulation and enzyme activity regulation and are unified under the control of the *ntr* regulon (Magasanik and Neidhardt, 1987).

'Nitrogen repression' of secondary metabolism refers to the inhibition of, reduction of, and/or delay in onset of, secondary metabolism by the presence of a readily catabolized nitrogen source (Brana and Demain, 1988). The nitrogen source represses secondary metabolism even in the presence of a second nitrogen source that does not cause repression. The systems of nitrogen repression of primary and secondary metabolism share another similarity in the type of repressor molecules (e.g. ammonium and some amino acids). It is important to note that the concentration of nitrogen source necessary to elicit secondary metabolite repression is usually very high; i.e. 10–120 mM.

The danger in the use of the term 'nitrogen repression' for secondary as well as primary metabolism is that the assumption may be made that the same regulatory mechanisms are responsible for both systems. 'Nitrogen repression', of secondary metabolism does not reflect a hierarchy of compound utilization, as the compound whose production is being repressed is not being further catabolized by the cell, whereas nitrogen repression of primary metabolism conserves the cell's resources. It is difficult to identify a purpose for nitrogen repression of secondary metabolism.

One is tempted to view nitrogen repression of secondary metabolism as an unfortunate consequence of metabolism rather than a specific mechanism of control of secondary metabolism; i.e. an essential secondary metabolite precursor is subject to nitrogen repression or inhibition. Such a possibility appears

to be the case in the synthesis of tylosin by *S. fradiae* (G68). The polyketide backbone (protylonolide) of this compound is derived from acetate, propionate and *n*-butyrate, which are derived from α-ketoisovalerate and other organic acids derived from amino acids. The enzyme that generates α-ketoisovalerate from valine is valine dehydrogenase (VDH). Ammonium is the other product of this reductive deamination, and so VDH is also an important enzyme of amino acid catabolism. It is perhaps not surprising, therefore, that VDH is subject to nitrogen repression. We might also expect high concentrations of ammonium to have an inhibitory effect on VDH, as it is a product of the reaction. A surprising observation was that VDH was subject to inorganic phosphate repression. As VDH was subject to nitrogen and phosphate repression and ammonium inhibition, it was to be expected that the secondary metabolite derived from VDH action, tylosin, was subject to nitrogen and phosphate repression. In confirmation of this mechanism, it was found that addition of acetate, propionate and *n*-butyrate to production media overcame ammonium repression of tylosin (Omura and Tanaka, 1986).

Amino acids are sources of carbon skeletons as well as ammonium. Therefore, we might expect that amino acid catabolism will be subject to the same modes of regulation as other carbon catabolites. This is certainly true in enteric bacteria where amino acid breakdown is subject to glucose repression (Magasanik and Neidhardt, 1987).

3.1. Proteases and Peptidases

Proteins are an important source of nitrogen, but they cannot be exploited without the intervention of proteases. Streptomycete proteases have been studied and the three-dimensional structures of several have been solved. Interest in streptomycete proteases has come from their potential for biotechnological application, rather than their role in the life cycle of the producing organism. It is not my intention to catalogue all the proteases and peptidases that have been identified in streptomycete culture filtrates. Rather I will attempt to relate what is known about the regulation of production and activity of proteases and peptidases and their possible role(s) in the streptomycete life cycle.

While streptomycete proteases and peptidases have received some attention, it is still the bacilli and fungi that supply most of the proteases used in commercial applications (Vitale *et al.*, 1980). A number of streptomycetes produce a complex of proteases and peptidases. Probably the best-characterized system is the 'pronase' complex from *S. griseus* K-1 (A1B). Some 12 different enzyme activities have been identified in this complex, including elastase, subtilisin, trypsin-like protease, chymotrypsin-like protease, metalloendopeptidases I and II, leucine peptidase, aminopeptidase and carboxypeptidase (Pokorny *et al.*, 1979; Tsuyuki *et al.*, 1991). Work has begun on the dissection of the protease-

peptidase complex produced by *S. rimosus* (B42). So far a serine protease, a trypsin-like protease, a leucine amino-peptidase and an arginine aminopeptidase have been resolved in culture filtrates from spent media used in oxytetracycline fermentations (Renko *et al.*, 1989).

3.1.1. Proteases

Detailed analysis at the molecular level of the *S. griseus* K-l (A1B) pronase complex has been in progress for several decades in a number of research groups around the world. The genes of two of the serine proteases, A and B, have been cloned and sequenced (Henderson *et al.*, 1987). This follows the amino acid sequencing of protease A (Johnson and Smillie, 1974) and protease B (Jurasek *et al.*, 1974), and the solution of their three-dimensional structures (Delbaere *et al.*, 1975; James *et al.*, 1980). Henderson *et al.* (1987) reported the presence of two distinct sequences at the N-termini of each open reading frame, which were not present in the mature protease. In each case the outermost sequence had the appearance of a signal sequence for protein export. The authors suggested that the sequence next to the signal sequence could either have a role in export beyond the cell wall (i.e. as a molecular chaperon), or inactivation of enzyme activity during synthesis and export. There was evidence that these sequences would be removed from the proprotease following autocleavage by the mature protease. Chang *et al.* (1990) also reported this kind of preproenzyme structure for the *S. cacaoi* (A16) extracellular metalloprotease. The amino acid sequence of the zinc-carboxy-peptidase purified from the *S. griseus* (A1B) pronase complex has also been reported (Narahashi, 1990). However, notwithstanding this detailed molecular information, very little has been published concerning the regulation of the activities of these biologically important extracellular proteins.

Shin and Lee (1986) reported some of the regulatory aspects of an extracellular alkaline protease from *Streptomyces* sp. SMF 301. The biosynthesis of the protease was significantly induced by skimmed milk and soya bean meal. It was not clear that protein was the true inducer as it is difficult to envisage how the cell perceives the presence of such large molecules. It should be noted that soya and casein hydrolysates were poor inducers, and so mixtures of peptides and amino acids either did not contain the inducer or there were components within the mixture that repressed the inductive ability of other components. Increased levels of induction were obtained following starvation for inorganic sulphur. Repression of protease biosynthesis occurred in media containing ammonium or some free amino acids. The best repressing amino acid was cysteine (3.5-fold). Methionine and lysine each repressed protease production 1.5-fold. Leucine caused mild stimulation (1.2-fold) of activity. The other amino acids tested (proline, serine and glutamate) had no effect. The

repressive effect of cysteine and methionine and the observation that sulphur starvation increased induction led the authors to suggest that the protease was a means by which the cell obtains sulphur-containing amino acids. The authors also reported that glucose was a repressive carbon source, but there was no inducer present in the medium. Also, fructose and lactose supported lower production of the protease in the salts medium.

The molecular basis of gene-specific regulation of protease production is now clearer. A number of small metalloprotease encoding genes have been isolated and sequenced from *S. coelicolor* (A1A) (Dammann and Wohlleben, 1992), *S. lividans* (A21) (Lichenstein *et al.*, 1992) and *Streptomyces* sp. C5 (Lampel *et al.*, 1992). In each case, an open reading frame (ORF) with homology to the LysR family of regulators was found upstream of, and divergently transcribed from, the protease gene. The product of the *S. coelicolor* (A1A) ORF bound to the protease gene promoter region and was essential for its activation in *S. lividans* (A21) and *E. coli*. The implication was drawn that this regulator bound a co-inducer that led to expression of the proteases. As the mechanism was operative in *E. coli*, it was further implied that the co-inducer was a readily available molecule; e.g. an amino acid or peptide (Dammann and Wohlleben, 1992).

Extracellular protease activity in *S. clavuligerus* (J71) appeared to be due to a single metalloprotease of 41.7 kDa, which was constitutive, not dependent on an exogenous inducer but suppressed at high cell growth rates. However, if fast-growing cells were subjected to a nutrient shift down, such as transfer from amino acid-rich medium to amino acid-poor medium, the protease was induced. Protease was also induced in untreated cells when they reached the end of the exponential growth phase. There was some evidence that amino acids supplied at low levels stimulated protease production. This growth rate-dependent control of protease production suggested a role for stringent response, and there was some evidence from use of partially relaxed mutants that this was the case (Bascaran *et al.*, 1990b).

There was evidence of nitrogen catabolite repression, in this case ammonium repression, of the protease, in addition to the growth rate control. The presence of ammonium in the medium suppressed total protease production by 4.6-fold. The repression was released in mutants of *S. clavuligerus* (J71) that also released ammonium repression of amino acid and urea catabolic enzymes. There were also mutants available in which repression of these catabolic enzymes was constitutive. Such mutants did not produce much protease even in the absence of ammonium. A similar situation was seen with the *S. spheroides* (A1B) protease(s) which was repressed by ammonium, nitrate and sulphate (Al-Nuri *et al.*, 1986); again protease production was constitutive and was not stimulated by addition of protein to the medium.

Aretz *et al.* (1989) reported the presence of three endo-proteases and two amino-peptidases in culture filtrates of *S. lividans* (A21), and that greatest

total proteolytic activity was associated with the late exponential phase of growth following glucose exhaustion, implying glucose repression of the protease–peptidase complex. Glucose repression of protease–peptidase complexes has also been reported in *S. spheroides* (A1B) (Al-Nuri *et al.*, 1986) and *S. aureofaciens* (A14) (Laluce and Molinari, 1977). Glucose repression was not universal. Gibb *et al.* (1989) reported that, in the A13 mutant of *Streptomyces* sp. C15, azocaseinase activities were not subject to physiologically significant glucose repression, but there was evidence that biosynthesis was associated with slow growth rate at the end of exponential growth. Ginther (1979) reported a similar phenomenon in *S. lactamdurans* (A7) and Gibb and Strohl (1988) in *S. peucetius* (?). It was suggested that this pattern of protease production was associated with release of primary metabolites for secondary metabolism and/or aerial mycelium formation and sporulation. Disler (1982) reported that a similar growth-phase regulated protease complex in *S. roseoflavus* var. *roseofungini* (?) could be suppressed by changes in the osmotic strength of the medium.

The production of the *S. aureofaciens* (A14) caseinolytic protease complex was dependent on the ratio between carbon source and nitrogen source. For maximal production, this ratio had to be high (Laluce and Molinari, 1977). This observation had been made with streptomycete protease complexes previously; e.g. in *S. rectus* var. *proteolyticus* (?) (Mizusawa *et al.*, 1969). It is not known how the streptomycete measures this ratio, but it is often reported that sporulation and secondary metabolite production are dependent on the ratio of carbon source to nitrogen source.

To summarize, there appears to be one class of proteases that is induced by protein catabolites and is subject to nitrogen catabolite repression. Another class of proteases is constitutive but regulated by growth rate and nitrogen and carbon catabolite repression. The appearance of a third class of proteases appears to be correlated with onset of cellular differentiation (i.e. spore formation).

3.1.2. Peptidases

The results of a survey of streptomycetes peptidase activities revealed that *S. citreus* (A1A), *S. flavochromogenes* (A5), *S. flavoviridis* (A28), *S. globisporus* (A1B), *S. halstedii* (A1C), *S. phaeochromogenes* (A40) and *S. oidiosporus* (?) all produced aminopeptidase activity alone. *S. filamentus* (A5?), *S. griseolus* (A1C) and *S. griseoflavus* (A37) produced only carboxy-peptidase activity, whilst *S. fradiae* (G68) and *S. peptidofaciens* (?) produced both types of activity. The latter strain produced the highest activity of both types of enzyme of all the streptomycetes tested, but it did not produce any detectable protease activity and so was investigated further. The greatest level of activity of both enzymes was found to be associated with the late exponential/stationary

growth phase of cells in batch culture. There was also a correlation between free sugar exhaustion and maximal production (Uwajima *et al.*, 1973). The above were all extracellular peptidases, but Vitale *et al.* (1996) reported the characteristics of an intracellular peptidase from *S. rimosus* (B42).

Vosbeck *et al.* (1978) reported some observations on the regulation of *S. griseus* K-l (A1B) aminopeptidase-I enzyme activity. Amino acids, especially histidine, were good inhibitors and both D- and L-amino acid isomers were equally effective. Evidence was also presented which implied that the binding site for the regulatory amino acids was different from the substrate-binding site. We might expect that the feedback regulatory role of the products of peptide cleavage has a biological function.

3.1.3. Protease Inhibitors

A wide range of protease inhibitors has been isolated from streptomycetes. Again the interest has often been pharmaceutical or biotechnological. In some cases the inhibitors have proved to be small, such as the tripeptide Leupeptin (Ning and Beynon, 1986), whilst in other instances the inhibitors have proven to be proteins, such as *Streptomyces* subtilisin inhibitor, which consists of a homodimer of a peptide of 11.5 kDa (Taguchi *et al.*, 1989). Little has been reported on the physiological role of these inhibitors in the streptomycete life cycle. Are they involved in reducing competition from other protease-producing organisms, so allowing the unreserved exploitation of protein resources by the inhibitor producer? If so, we would expect the inhibitor producer's own proteases to be resistant to the inhibitor. This is not always the case. Another possibility is that the inhibitors are a means by which the cell can synthesize and export proteases without the danger of autolysis. We need further work before we can judge what are the roles of these inhibitors in nature. When are they produced and under what conditions? How effective are they at inhibiting proteases produced by the same cell?

3.2. Peptide and Amino Acid Transport

3.2.1. Peptide Transport

Little has been reported concerning peptide transport in streptomycetes. This is surprising considering that in all probability most of the cell's nitrogen will be ultimately derived from protein that has been solubilized by proteases. We might expect that in most cases the initial products of protease action will be short-chain peptides rather than free amino acids. It would therefore be surprising if streptomycetes cannot transport peptides when so many other

bacteria and many fungi can (Matthews and Payne, 1980). It would seem axiomatic to suggest that a cell with the ability to transport peptides as well as amino acids would have a competitive advantage over a cell that has to wait for amino acids to be released by the action of peptidases on peptides.

Leigh (1992) has demonstrated the presence of two peptide transport systems in *S. coelicolor* A3(2) (A21). Using a mutant unable to transport proline (*put*⁻), he was able to show that the cell could grow on L-prolyl-proline and L-prolyl-prolyl-proline, but not on proline as the sole carbon and energy source. Use of ^{3}H- and ^{14}C-labelled proline peptides confirmed the cell could transport these molecules. Using a number of small peptide antibiotics Leigh was able to isolate mutants that could not transport peptides. Cells resistant to bialaphos (Diddens *et al.*, 1976) could not transport dipeptides, whilst those resistant to Ro3-9699 could not transport larger peptides and hydrophobic dipeptides. Double mutants resistant to both drugs could not transport any peptides assayed. Such mutants could, however, grow on peptides containing amino acids other than proline. This result was evidence for the presence of peptidases that cleave the peptides to release the amino acids that can be transported directly into the cell, as reported by Vitale *et al.* (1996).

The bialaphos-transporting peptide transport system was characterized in the same strain. A bialaphos-resistant transposon mutant was shown to contain a transposon insertion in a cluster of five genes encoding a typical ABC transporter with homology to oligopeptide permeases in other bacteria. The transposon insertion and a deletion engineered in the same gene cluster had a bald phenotype. This led to the genes being designated *bldK* (Nodwell *et al.*, 1996). The *bldK* mutants were believed to be bald because they could not import an essential developmental peptide signal (Nodwell and Losick, 1998). The peptide transport capacity of the *bldK* strains was not assayed. Surprisingly, the bialaphos-resistant, Ro3-9699-resistant double mutants of Leigh (1992) that were fully deficient in peptide transport were not notably deficient in aerial mycelium formation.

3.2.2. Amino Acid Transport

Ring and Gross subjected the amino acid transport systems of *S. hydrogenans* (A5) to extensive and rigorous study and concluded that there are at least four different amino acid permeases in this strain: a neutral amino acid permease; an arginine-specific permease; a basic amino acid permease; and an acidic amino acid permease (Table 11). This table also summarizes the information on other amino acid permeases of streptomycetes.

3.2.2.1. Neutral Amino Acid Permeases. Using the non-metabolized amino acid analogue α-aminoisobutyric acid (AIB), Ring and Heinz (1966)

Table 11 Amino acid transport permeases in streptomycetes.

Strain	System and Substrates	K_m(μM)	V_{max}(μmol. min^{-1} (g protein)$^{-1}$	Reference
S. antibioticus (A31)	*Glutamate-induced*			
	Glutamate	67	71.4	
	Proline	28	17.0	May and Formica, 1978
	Constitutive			
	Glutamate	40–67	2.5–22.2	
	Proline	28–36	2.2–2.5	
S. clavuligerus (J71)	*Proline-specific*			
	Proline	11	9	Bascaran *et al.*, 1990a
	Non-specific			
	Proline	10 200	112	
S. hydrogenans (A5)	*Neutral amino acids*			Ring and Heinz, 1966
	α-aminoisobutyrate	38	29	
	Arginine	700	2.5	
	Aspartate (pH 6.5)	71	3.7	
	Aspartate (pH 4.5)	110	2.9	

Table 11 *cont.*

Strain	System and Substrates	K_m(μM)	V_{max}(μmol. min^{-1} (g protein)$^{-1}$	Reference
	Glutamate	2000	25	Gross and Ring, 1971
	Lysine	1600	3.5	
	Arginine-specific			Gross and Burkhardt, 1973
	Arginine	7	0.6	
	Basic amino acids			
	Arginine	0.6	0.01	
	Lysine	0.9	0.05	
	Acidic amino acids			Ring *et al.*, 1977b
	Aspartate (pH 6.5)	3.9	0.67	
	Glutamate	10	2.0	

demonstrated that resting cells could concentrate the substrate 200-fold. The process required active respiration. The affinity (K_m) of the permease for the substrate was in the μM range (38 μM) in line with amino acid permeases of other bacteria. As discussed above (section 2.2.3), constitutive carbohydrate transport systems of streptomycetes have low affinity (K_m in the mM range) for their substrate. There was no indication that neutral amino acid transport was driven by Na^+ ions; indeed, Na^+ inhibited transport by competing with the amino acid for the binding site. This competitive inhibition was seen with other alkali ions with decreasing effectiveness in the following order: $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ (Ring *et al.*, 1976).

Pre-loading of the cells with AIB led to a decrease in the rate of AIB transport, but it did not affect the affinity of the permease for AIB; i.e. there was feedback inhibition of substrate transport (Ring and Heinz, 1966). Pre-loading of the cell with any neutral amino acid led to the *trans*-inhibition of transport of other amino acids. However, pre-loading of the cells with lysine, proline, glutamate or aspartate *trans*-stimulated the transport of all the amino acids tested, including AIB. *Trans*-stimulation took place after a 20-minute lag period, whilst *trans*-inhibition was immediate and did not affect transport of ions (K^+) or carbohydrate (sorbose). Further kinetic experiments demonstrated that both *trans*-stimulation and *trans*-inhibition affected the rate, but not the affinity, of the transport systems and implied that the effect was mediated by the alteration of the energy coupling of the permease (Gross *et al.*, 1970; Ring *et al.*, 1970).

The inability of chloramphenicol to stop *trans*-stimulation confirmed that this process did not affect gene activation. The nature of the molecule mediating the *trans*-stimulation effect was postulated to be an intermediate in amino acid metabolism with an intact amide group. Langheinrich and Ring (1976) speculated that the intermediate was a charged tRNA. However, they did not present evidence to support this proposal.

Gross and Ring (1969) reported that chloramphenicol reduced the level of AIB transport by 50% after 20 minutes of treatment, which implied that the AIB transport system was rapidly turned over. AIB, alanine or glycine induced the neutral amino acid permease. In the presence of other amino acids that in themselves had no inducing power, the induction of the permease by a true inducer was enhanced. In particular, asparagine enhanced induction by alanine by more than four-fold (Ring, 1969).

Amino acid transport was dependent on the growth rate and growth phase of cells in a culture. The greater the growth rate, the greater the capacity of the cells for amino acid transport. In part, the growth phase-dependency was determined by the size of the intracellular amino acid pools and independent of the presence or absence of amino acids in the growth medium. It was concluded that the amino acid transport systems were mediated by feedback regulation by intracellular amino acid pools that affected both permease synthesis and

efficiency (Alim and Ring, 1976; Langheinrich and Ring, 1976). Further work (Ring *et al.*, 1977a, c) implicated a role for cyclic nucleotides in the regulation of amino acid permease induction. Exogenously supplied dibutyryl cGMP and dibutyryl cAMP both led to stimulation of permease synthesis over a period of 30–60 min. This synthesis was inhibited by rifampicin, the RNA polymerase inhibitor. The cyclic nucleotides had no effect on the glycerol, sorbose or pyrimidine transport systems, so the effect was presumed to be specific to amino acid permeases. It is tempting to speculate that the growth cycle/growth rate dependence of amino acid transport was mediated by cyclic nucleotides.

S. lipmanii (A7) contains threonine, isoleucine and valine permease activities that are inhibited by leucine. It is tempting to suggest that there is one transport system for all four amino acids and the inhibition observed is competition for the common permease. This discovery explained the observation that threonine auxotrophs were sensitive to leucine on fully supplemented media (Kirkpatrick and Godfrey, 1973).

3.2.2.2. Basic Amino Acid Permeases. *S. hydrogenans* (A5) contains two energy-dependent systems for transporting basic amino acids (Gross and Burkhardt, 1973): system b_{Arg}, is highly specific for arginine, whilst system $b_{Arg/Lys}$ can transport either arginine or lysine (Table 11). Evidence was presented that system $b_{Arg/Lys}$ was induced by arginine and lysine whilst system b_{Arg} was constitutive. The transport capacity of both these systems was very poor, but the affinity was high. The affinity of system $b_{Arg/Lys}$ for arginine was higher than system b_{Arg}, but the V_{max} was ten-fold lower. At high substrate levels (i.e. in the mM range), both lysine and arginine could be transported on the neutral amino acid permease at far greater rates than on the specific systems. The authors calculated that at high substrate concentration the neutral amino acid permease was responsible for the influx of 75% of the arginine and 99% of the lysine found in the cell.

3.2.2.3. Acidic Amino Acid Permeases. In *S. hydrogenans* (A5), acidic amino acids were transported either by a specific energy-dependent permease, which transported both aspartate and glutamate in their anionic form, or by the neutral amino acid permease, which could transport these amino acids in only their zwitterionic forms. Hence, the transport system used depended on the pH of the growth medium. The acidic amino acid permease had a higher affinity for both acidic amino acids, but the neutral amino acid permease could transport them at greater rates (Table 11) (Gross and Ring, 1971; Ring *et al.*, 1977b; Fritsch and Gross, 1983).

3.2.2.4. Proline Permeases. In *S. antibioticus* (A31), proline could be transported either by an energy-dependent, constitutive, proline-specific permease, or by an energy-dependent, glutamate-induced, non-specific

permease (Table 11). Glutamate, on the other hand, could be transported only on the glutamate-induced, non-specific permease. Surprisingly the pH optimum of glutamate transport was 7.0–8.0, which implied that the glutamate-induced, non-specific permease could transport glutamate in its anionic form and other amino acids in an uncharged form (May and Formica, 1978).

There were two active proline transport systems in *S. clavuligerus* (J71) (Table 11). One system had high affinity for proline but low capacity and proline transport was out-competed only by alanine. The second proline transport system had low affinity but high capacity and appeared to be a general amino acid permease as six of the seven amino acids tested out-competed proline transport. When proline was present in the culture at 0.1 mM, 87% of proline transport was via the high-affinity system. When 5mM proline was present in the culture medium, 82% of proline transport was via the low-affinity system. Both systems were constitutive and neither was subject to repression or inhibition by ammonium. Unlike the system in *S. antibioticus* (A31), glutamate had no stimulating effect on either system (Bascaran *et al.*, 1990a). The proline transport system of *S. venezuelae* (A6) has also been examined indirectly. There was evidence that ammonium and nitrate delayed proline uptake (Shapiro and Vining, 1984).

In *S. coelicolor* A3(2) (A21), proline transport was constitutive and was not repressed by ammonium or glucose. Mutants unable to transport proline were isolated as a subclass of mutants resistant to a number of proline analogues. The inability of these *put*⁻ strains to transport the amino acid was confirmed by the use of ^{14}C proline. It should be noted that all these mutants retained the ability to transport all other amino acids. One surprising observation was that all these *put*⁻ mutants overproduced undecylprodigiosin, one of the secondary metabolites of *S. coelicolor* A3(2) (A21). This was thought to be a consequence of the fact that proline is a direct precursor of undecylprodigiosin and that the *put*⁻ mutants have also lost the ability to degrade proline (Hood *et al.*, 1992).

3.3. Amino Acid Catabolism

3.3.1. The Aromatic Amino Acid Family

3.3.1.1. Tryptophan. As discussed below (see section 4.1.1.4), tryptophan, in addition to its role in protein synthesis, is a precursor of NAD^+ biosynthesis in *S. antibioticus* (A31). This role was not universal in streptomycetes, as it was not so used by *S. venezuelae* (A6) (Lingens and Vollprecht, 1964). Tryptophan is also important as the precursor of a number of streptomycete secondary metabolites, such as actinomycin D (Hitchcock and Katz,

1988) and streptonigrin (Hartley and Speedie, 1984). This amino acid is also a potential carbon and nitrogen source.

Tryptophan was catabolized in all streptomycetes tested (Teuscher, 1967). However, only *S. exfoliatus* (A5) and two uncharacterized streptomycetes were capable of degrading it completely. The majority of strains – i.e. *S. limosus* (A1A), *S. coelicolor* (A1A), *S. cremeus* (A1B), *S. cyaneofuscatus* (A1B), *S. flavovirens* (A1C), *S. griseus* (A1B), *S. flaveolus* (A24), *S. massasporeus* (D) and *S. bobiliae* (?) – accumulated anthranilate and formyl-anthranilate.

The pathway of tryptophan catabolism has been characterized in *S. parvulus* (A12), the producer of actinomycin D (Katz *et al.*, 1984). The pathway proceeded via formyl-kynurenine, kynurenine, hydroxy-kynurenine and hydroxy-anthranilate. The first enzyme of the pathway, tryptophan dioxygenase, was purified and characterized and was neither induced nor repressed by tryptophan. However, activity was induced 40-fold during the antibiotic production phase. This induction was due to release of repression of the enzyme by D- or L-glutamate in the medium. D- or L-aspartate or L-alanine repressed the enzyme by greater than 72%. Other amino acids had lesser repressive effects. NAD^+, $NADP^+$, NADH, NADPH, anthranilate and kynurenine had no effect on enzyme activity, whilst hydroxy-kynurenine caused 60% inhibition via competitive inhibition. Hydroxy-anthranilate also inhibited activity, but this may have been via an indirect effect as the amount of inhibition increased with time of incubation (Foster and Katz, 1981; Hitchcock and Katz, 1988).

Two forms of the second enzyme of the pathway, kynurenine formamidase, were found in *S. parvulus* (A12). One isozyme (kynurenine formamidase I) was found to be constitutive and to be present at low levels, whilst the second enzyme appeared to be induced during production of actinomycin D. This implied that the former isozyme was responsible for tryptophan breakdown/NAD synthesis, whilst the latter was induced as part of the actinomycin D biosynthetic pathway. An enzyme similar to kynurenine formamidase I isozyme was also found in *S. antibioticus* (A31). Kynurenine formamidase of equivalent specific activity was found in a number of streptomycetes – i.e. *S. avermitilis* (?), *S. caespitosus* (F58), *S. cattleya* (C47), *S. clavuligerus* (J71), *S. coelicolor* (A1A), *S. griseoviridis* (A17), *S. griseus* (A1B), *S. kanamyceticus* (B42) and *S. lividans* (A21) (Brown *et al.*, 1986).

Two other enzymes from tryptophan degradation pathways have been examined in *S. parvulus* (A12). Kynureninase activity, which converts kynurenine to anthranilate, was found to increase by 35-fold in the presence of tryptophan, whilst hydroxy-kynureninase activity, which converts 3-hydroxykynurenine to 3-hydroxyanthranilate, was found to increase 18-fold. 3-Hydroxyanthranilate is an important precursor of actinomycin and NAD^+, if that pathway was present. Hydroxy-kynureninase activity increased (50 to 60-fold) during actinomycin synthesis (Troost *et al.*, 1980). It was not clear whether the

enzyme had functions in either amino acid catabolism or secondary metabolism, or both.

3.3.1.2. Phenylalanine and Tyrosine. Phenylalanine ammonia-lyase (PAL), the enzyme responsible for non-oxidative deamination of the amino acid to cinnamate, was present in *S. verticillatus* (?). It was specific for phenylalanine, being unable to use tyrosine as substrate (Emes and Vining, 1970). Cinnamamide, a derivative of cinnamate, was accumulated only in the presence of excess phenylalanine, which implied that the streptomycete could not catabolize phenylalanine to release usable energy and carbon (Bezanson *et al.*, 1970). It is difficult to assign a catabolic role for cinnamide as it consumes the amine released by PAL.

S. setonii (A1B) was the only streptomycete tested that completely metabolized phenylalanine and tyrosine. The pathway of metabolism was via homogentisate and no evidence of phenylalanine 4-hydroxylase could be found; i.e. phenylalanine was not degraded via tyrosine. *S. viridosporus* (A1B) could degrade phenylalanine to phenylacetate but not to homogentisate. None of the other streptomycetes tested, *S. sioyaensis* (A29), *S. badius* (C) and *Streptomyces* sp. V7, could metabolize phenylalanine. Surprisingly all five strains could induce homogentisate oxygenase with phenylalanine and tyrosine even though four of the strains could not produce homogentisate. The enzyme was induced by either amino acid in *S. badius* (C), *S. setonii* (A1B) and *S. sioyaensis* (A29), by phenylalanine alone in *S. viridosporus* (A1B) and by tyrosine alone in an uncharacterized *Streptomyces* sp. (Pometto and Crawford, 1985). Tyrosine could be catabolized to yield enough carbon and nitrogen to support growth by *S. michiganensis* (A6) and the pathway of catabolism was again via homogentisate (Held and Kutzner, 1988).

Another major sink for tyrosine in streptomycetes is the formation of melanin by tyrosinase. It is not clear why streptomycetes possess tyrosinase but many do, i.e., up to one third of streptomycetes isolated from soil (Kuster, 1976). It has been suggested that melanin deposited within fungal cell walls has an inhibitory effect on cell lytic enzyme produced by bacteria and other fungi (Kuster, 1976). In some cases streptomycete tyrosinases were tightly regulated. The *S. glaucescens* (A28) enzyme was inducible by methionine, leucine or phenylalanine, but not by tyrosine (Crameri *et al.*, 1984). Methionine, leucine and phenylalanine and nor-valine induced the enzyme of *S. antibioticus* (A31). However, only methionine induced transcription of the gene encoding the enzyme at low concentration (0.1 mM). One hundred-fold more inducer was needed for the other amino acids (Katz and Betancourt, 1988; Betancourt *et al.*, 1992). The tyrosinase of *S. michiganensis* (A6) was induced at the level of transcription by copper and repressed by ammonium (Held and Kutzner, 1990). Leu *et al.* (1989) and Geistlich *et al.* (1989) have reported the beginnings of the molecular analysis of the promoters of the tyrosinase genes of *S. antibioticus* (A31) and *S. glaucescens* (A28) respectively.

3.3.2. Histidine

Histidine catabolism has been studied in depth in two streptomycetes. In *S. coelicolor* A3(2) (A21) it proceeded via formylglutamic acid. This pathway was also found in pseudomonads, but not in enteric bacteria or the bacilli. The *hut* system appeared to be inducible and was repressed by neither ammonium nor glucose. Circumstantial evidence was presented that urocanate, the product of histidase action on histidine, was the true inducer of the system (Kendrick and Wheelis, 1982). The histidases of *S. griseus* (A15) and *S. coelicolor* A3(2) (A21) were regulated at the post-translational level. From biochemical studies and analysis of mutants it appeared that the streptomycete *hut* system, excluding the histidase gene, was induced at the transcriptional level by urocanate. The enzyme histidase was subject to control by two proteins: activation factor and inactivation factor. Activation factor could be replaced, *in vitro*, by snake venom phosphodiesterase, which implied that histidase was inactivated by adenylation. Activation factor was regulated at the enzyme level; i.e. inactivation by phosphorylation. The data were consistent with the proposal that urocanate acted as an activator of phosphorylated activation factor phosphatase, i.e. urocanate was responsible for the activation of histidase, via the activation factor, and induction of the rest of the *hut* genes (Kroening and Kendrick, 1987, 1989). Why streptomycetes should have this dual control of histidine catabolism is a question that tempts speculations. However, we will resist the temptation.

The *S. griseus* (A15) histidase was activated as cells entered the stationary phase, but was inactivated when cells were induced to sporulate by either phosphate starvation or nutrient downshift. Nutrient replenishment reversed the latter effect (Kroening and Kendrick, 1987).

The histidase of *S. griseus* (A15) has been purified to homogeneity and used to clone the gene, *hutH*. Kinetic studies of the purified enzyme revealed differences with other bacterial and eukaryotic histidases. The streptomycete enzyme had an eight-fold lower K_m (higher affinity) for its substrate than other bacterial histidases, and D-histidine and histidinol phosphate competitively inhibited the enzyme. The latter compound did not inhibit other histidases. The *hutH* gene was sequenced and shown to be similar to both bacterial and eukaryotic histidases however, as implied from the kinetic data, it had diverged from the previously characterized histidase family. Evidence was presented that the enzyme was translated from a leaderless mRNA; i.e. there was no ribosome-binding site. The translation of leaderless transcripts has been identified in a number of other streptomycete systems (Jones *et al.*, 1992). Putative promoter sequences were identified by consensus sequence homology; and multicopy, plasmid-based, promoter-probe studies implied that the *hutH* promoter was constitutive (Wu *et al.*, 1992a, b). However, later work corrected this implication as *hutH* was subject to negative regulation, because multiple copies of the promoter region alone led to derepression of the gene (Wu *et al.*, 1995).

Histidase and urocanase were induced by the presence of histidine in the growth medium of *S. clavuligerus* (J71) (Bascaran *et al.*, 1989a). As in *S. griseus* (A1B) and *S. coelicolor* A3(2) (A21), ammonium did not repress either enzyme.

Histidine aminotransferase, a potential histidine degradative enzyme, was identified in a *hut* mutant of *S. tendae* (A12) that lacked histidase activity. The enzyme was specific for histidine and was capable of transferring an amino group to pyruvate to form alanine. 2-oxobutyrate, 2-oxovalerate and 2-oxo-caproate were also used as keto acceptors by the purified enzyme. Histidine aminotransferase did not appear to be part of an alternative histidine catabolic pathway as the *hut* strain could not use histidine as a sole carbon and nitrogen source. It was not clear if the enzyme allowed use of histidine as the sole nitrogen source. The kinetics of enzyme production during the growth cycle implied that it was involved in nikkomycin biosynthesis (Roos *et al.*, 1992; Roos and Bormann, 1993).

3.3.3. The Glutamate Family of Amino Acids

Glutamate and glutamine are special cases in that in streptomycetes, as in other bacteria, they are the main routes of nitrogen assimilation. Therefore, the discussion of glutamate and glutamine catabolism is more logically discussed in the section on nitrogen assimilation (see section 3.6).

3.3.3.1. Proline. The pathway of proline catabolism involves two enzymes: proline oxidase, which converts proline to Δ′pyrroline-5-carboxylate (P5C); and pyrroline-5-carboxylate dehydrogenase (P5CDH), which converts glutamate-4-semialdehyde, the spontaneous product of pyrroline 5-carboxylate hydration, to glutamate. This pathway is operative in *S. coelicolor* A3(2) (A21) (Hood *et al.*, 1992; Smith *et al.*, 1995). In enteric bacteria both enzyme activities are fused in a single peptide, but in this streptomycete the enzyme functions were separate. Proline oxidase activity was membrane-bound whilst P5CDH was found in the cytoplasm. Proline induced P5CDH activity 300-fold at the level of transcription (Smith *et al.*, 1995). The enzymes activities were not repressed by growth in the presence of either ammonium or glucose.

There was evidence that in *S. niveus* (A1B) proline catabolism was repressed by ammonium (Kominek, 1972), whilst the proline oxidase of *S. clavuligerus* (J71) was shown to be induced by proline, but not subject to ammonium repression (Bascaran *et al.*, 1989a). It would appear, therefore, that the nature of the regulation of proline catabolism was strain-specific. It might be relevant to note that *S. coelicolor* A3(2) (A21) produces a proline-derived secondary metabolite, undecylprodigiosin, whilst, to my knowledge, *S. niveus* (A1B) and *S. clavuligerus* (J71) do not. Strains of *S. coelicolor* A3(2) (A21)

that have lost the ability to degrade proline (*put* mutants) overproduced undecylprodigiosin. The implication is that if proline could not be removed from the cell it was diverted into secondary metabolism (Hood *et al.*, 1992).

3.3.3.2. Arginine. Arginine metabolism has received attention in streptomycetes because it is a precursor of a number of commercially important secondary metabolites; e.g. streptomycin and clavulanic acid. Bascaran *et al.* (1989a) reported the presence of arginase and ornithine aminotransferase (OAT) in *S. clavuligerus* (J71). This implied that arginine was degraded to glutamate and urea via ornithine and pyrroline 5-carboxylate. The complete pathway would be: arginase, OAT, and pyrroline 5-carboxylate dehydrogenase. The OAT was constitutive, but repressed by both ammonium and aspartate whilst arginase was induced by arginine and subject to ammonium repression. Surprisingly, Romero *et al.* (1986) reported that, whilst this same strain could use proline as sole carbon and nitrogen source, it could only use arginine as sole nitrogen source. Thus, there appeared to be a contradiction, as glutamate was the common product of both amino acid catabolic pathways. It either implied that the pyrroline 5-carboxylate dehydrogenase was not present in arginine-grown cells or that ornithine had an alternative destination and the urea generated by arginase action was being used as the nitrogen source. These authors reported that ornithine was efficiently incorporated into clavulanic acid. The purified OAT of *S. clavuligerus* (J71) had an associated arginase activity (de la Fuente *et al.*, 1996). These authors also showed that the enzyme was arginine-repressed but not inhibited.

S. griseus (A1B) could catabolize arginine, which it could use as both carbon and nitrogen source, via a different route from that of *S. clavuligerus* (J71). The pathway was: arginine, γ-guanidobutyramide, γ-guanidinobutyrate, aminobutyrate and succinate (Thoai *et al.*, 1962). Later work revealed that this was the sole pathway of arginine catabolism in the organism, that it was induced by arginine, and that the enzymes were induced to different levels (Thoai *et al.*, 1966). The first enzyme of the pathway, arginine oxidase (decarboxylating), was purified and shown to contain FAD and to release CO_2 (Olomucki *et al.*, 1968). Recently, Padilla *et al.* (1991) reported the presence of the third enzyme, guanidinobutyrate ureohydrolase, in all streptomycetes tested. The enzyme was induced by arginine: 20-fold in *S. lividans* (A21); 16.3-fold in *S. griseus* (A1B); and 32-fold in *S. clavuligerus* (J71). There was only a 1.8-fold induction of the enzyme in *S. coelicolor* A3(2) strain M130, but this appeared to be a strain-specific aberration and other strains showed much higher levels of activity in arginine media. Arginase was also present in all streptomycetes tested, although at low levels, and induction by arginine was less than 2.7-fold. The OAT of *S. coelicolor* A3(2) has been shown to have a similar structure to the *S. clavuligerus* (J71) enzyme (de la

Fuente *et al.*, 1996). The implication is that arginase and OAT of *S. clavuligerus* (J71) were involved in ornithine supply to secondary (clavulanic acid) metabolism.

Another route for arginine catabolism was proposed for the streptomycin-producing streptomycetes *S. griseus* (A1B), *S. griseocarneus* (?) and *S. bikiniensis* (A1B) (Walker and Hnilica, 1964). In these strains an amidinotransferase was responsible for the formation of ornithine from arginine by transferring the amidino group to inosamine, a precursor of streptomycin. Presumably this enzyme should be regarded as an enzyme of secondary metabolism rather that primary metabolism.

A similar situation arose in another streptomycete, *S. eurocidicus* (?), in which an unusual fate for arginine was discovered (Nakane *et al.*, 1977). Arginine induced a pathway that converted arginine to 2-oxo-4-hydroxyarginine, releasing ammonium and requiring molecular oxygen. This intermediate was further converted to 2-aminoimidazole with the release of pyruvate and water. The final product, 2-nitroimidazole, was the result of the oxidation of 2-aminoimidazole. It was not clear what the fates of the pyruvate and ammonium were. Were they incorporated into general metabolism? Was this a mechanism to catabolize arginine with the release of a carbon and energy source and a nitrogen source with a concomitant waste product, 2-nitroimidazole? Or was it a mechanism for synthesizing a secondary metabolite with an as yet unknown function? These questions address the fundamental aspects of the role(s) of secondary metabolism.

3.3.4. The Branched-Chain Amino Acids

3.3.4.1. Valine Dehydrogenase. A lot of interest has centred on the catabolism of branched-chain amino acids in streptomycetes because it proceeds via intermediates that are precursors to a wide range of polyketide and polyether antibiotics (Reynolds *et al.*, 1988). It was expected that an understanding of the regulation of catabolism (i.e. precursor supply) would lead to an understanding of the regulation of secondary metabolism.

The catabolic enzyme that has been studied most extensively in a wide range of streptomycete species is the first enzyme of the pathway, valine dehydrogenase (VDH). This enzyme is responsible for the oxidative deamination of all the branched-chain amino acids (valine, leucine and isoleucine) and a number of their analogues. VDH has been purified from *S. aureofaciens* (A14) (Vancurova *et al.*, 1988c), *S. cinnamonensis* (?) (Priestley and Robinson, 1989), *S. coelicolor* A3(2) (A21) (Navarrete *et al.*, 1990), and *S. fradiae* (G68) (Vancura *et al.*, 1988a), and a surprisingly

large number of differences have been noted. The physical structures of the *S. coelicolor* A3(2) (A21) and *S. cinnamonensis* (?) enzymes were rather similar but the other two were very different. The specific activity of *S. fradiae* (G68) VDH was more than 1000-fold greater than that of the *S. coelicolor* A3(2) (A21) or *S. cinnamonensis* (?) enzyme. The affinity for the substrates varied over a large range between the four enzymes, but it appeared that all four could deaminate all three branched-chain amino acids. However, the preferred amino acid was valine in all cases. It was also noted that NAD^+ was the preferred hydrogen acceptor and that $NADP^+$ was a poor substitute in all four enzymes.

Regulation of expression of the enzyme has been reported for a number of strains. *S. cinnamonensis* (?) VDH was induced greater than 100-fold by the addition of valine to the growth medium. No effects of glucose or nitrogen repression were reported (Priestley and Robinson, 1989). Disruption of the gene encoding the VDH in this streptomycete yielded a mutant that had no VDH activity but was still able to grow on valine as a nitrogen source and produce monensin. There was evidence of a pyridoxal phosphate-dependent valine transaminase in the strain that may substitute for the VDH activity (Leiser *et al.*, 1996).

Valine induced *S. aureofaciens* (A14) VDH by around ten-fold, whilst isoleucine induced it about eight-fold. Ammonium induced it approximately five-fold when compared with expression on medium containing alanine, asparagine, aspartate, glutamate or glutamine as sole nitrogen source (Vancurova *et al.*, 1988c). 17.8-fold valine induction still occurred in the presence of 100 mM ammonium (Nguyen *et al.*, 1995a). A second VDH activity has been purified from *S. aureofaciens* (A14). It has a different subunit size and arrangement to the enzyme purified by Vancurova *et al.* (1988c) and is valine-induced; it is claimed to be ammonium-repressed but again there was no inducer present (Nguyen *et al.*, 1995b).

S. fradiae (G68) VDH was induced 6.2-fold by valine, 3.7-fold by leucine, and 3.5-fold by isoleucine. In all cases the medium also contained 25 mM ammonium and so the effect of ammonium on induction cannot be assessed. No investigations were made into the repressive effects, if any, of the carbon source (Vancura *et al.*, 1989a). A more systematic study revealed 100 mM ammonium to have no effect on 8.6-fold valine induction of VDH (Nguyen *et al.*, 1995a). A second VDH activity was also purified from *S. fradiae* (G68). As with the second activity in *S. aureofaciens* (A14), the second enzyme differed from the first activity in subunit size and number and looked much more like the *S. cinnamonensis* (?) and *S. coelicolor* A3(2) (A21) enzymes. The second activity was valine-induced but the claim for ammonium repression was not substantiated by the data quoted, as ammonium had little effect if the inducer, valine, was present (Nguyen *et al.*, 1995c). Tang *et al.* (1994) reported the inactivation of a *S. fradiae* (G68) gene encoding VDH with great similarity to the

S. coelicolor A3(2) (A21) *vdh* gene. All VDH activity in the cell was lost and yields of tylosin were reduced.

The effect of ammonium on the VDH of *S. fradiae* in cells cultured in the absence of valine was assessed. Increasing ammonium concentration led to suppression of VDH activity: 5.1 to 5.8-fold from 25 mM to 100 mM (Omura *et al.*, 1983b; Vancura *et al.*, 1987). Addition of 25 mM and 100 mM ammonium to VDH *in vitro* led to 50% and 100% inhibition of the enzyme activity, respectively (Omura *et al.*, 1983b). It is difficult to differentiate between the effect of ammonium on gene expression and on enzyme activity. The fact that phosphate also decreased VDH activity led to the suggestion that the basis of phosphate and ammonium 'repression' of tylosin production was due to the decrease in availability of the precursor of the protylonolide portion of the antibiotic. This hypothesis was strengthened when it was discovered that addition of *n*-butyrate, propionate and acetate overcame phosphate and ammonium 'repression' (Omura *et al.*, 1984a, b). Evidence had previously been presented that α-ketoisovalerate, the product of VDH action on valine, was decarboxylated to form *iso*-butyrate that was then converted to *n*-butyrate before incorporation into protylonolide (Omura *et al.*, 1983a).

When asparagine or ammonium was available as the sole nitrogen source, *S. coelicolor* A3(2) (A21) VDH activity was below the detection limit of the assay; i.e. < 0.7 mU (mg protein)$^{-1}$. Histidine induced VDH more than 19-fold and valine more than 37-fold in the presence of glucose. When glycerol, a non-repressing carbon catabolite, was substituted for glucose the fold of induction by valine was increased to over 129. When valine was the sole carbon and nitrogen source, VDH increased at least 260-fold over glucose plus ammonium grown cells. It appears, therefore, that valine induced the enzyme but that induction was subject to glucose repression. It is not possible to state if the enzyme was subject to ammonium repression as the authors did not report the effect on cells of ammonium in the presence of the inducer, valine (Navarrete *et al.*, 1990). The *vdh* gene was cloned and disrupted in *S. coelicolor* A3(2) (A21). The *vdh* mutants could not grow on valine, isoleucine or leucine as sole nitrogen source (Tang and Hutchinson, 1993). Examination of *vdh* gene transcription revealed it to be induced by valine, and addition of glucose or ammonium in the presence of the inducer repressed expression. The glucose repression was dependent on the *glkA* gene (Section 2.4.5.4.) (Tang and Hutchinson, 1995).

No branched-chain amino acid dehydrogenase activity could be found in cells of *S. avermitilis* (?) cultured with either ammonium or isoleucine as sole nitrogen source. However, a branched-chain amino acid transaminase activity was present and evidence was presented that it included the action of more than one enzyme. The isoleucine transaminase was suppressed two-fold in cells grown on isoleucine as sole nitrogen source (Novak *et al.*, 1992b), which

might imply that the transaminase was involved in isoleucine synthesis rather than catabolism. Nguyen *et al.* (1995d) reported they could find VDH in *S. avermitilis* although at lower activity than the transaminase of Novak *et al.* (1992b). They reported that the VDH was induced five-fold by valine and claimed it was ammonium-repressed. However, they did not report the effect of ammonium when inducer was present. The acetylation of isoleucine to form *N*-acetyl-isoleucine was also reported in *S. avermitilis* (?) (Nemecek *et al.*, 1992). It was proposed that this was a mechanism to detoxify isoleucine, which inhibited growth. The acetylated amino acid at first accumulated in the culture medium, but was eventually converted into α-keto-β-methylvalerate, the deaminated metabolite of isoleucine. This implied that *N*-acetyl-isoleucine was a substrate of isoleucine catabolism.

Valine-induced and ammonium-repressed VDH has been shown to be important for precursor supply to spiramycin in *S. ambofaciens* (A23), as addition of valine stimulated antibiotic production, and ammonium and glycerol addition suppressed VDH activity and antibiotic formation. Adding the products of VDH activity could negate this suppression. However, ammonium repression of VDH activity was not the only reason for ammonium repression of spiramycin biosynthesis (Laakel *et al.*, 1994; Lounes *et al.*, 1995a, b). Disruption of the *S. ambofaciens* (A23) *vdh* gene led to loss of spiramycin titre (Tang *et al.*, 1994).

3.3.4.2. α-Keto Acid Dehydrogenase. The second enzyme in the branched-chain amino acid catabolism pathways is an α-keto acid dehydrogenase. This enzyme carries out the oxidative decarboxylation of the branched-chain 2-oxo acid with the concomitant addition of coenzyme A. A mutant of *S. avermitilis* (?) has been isolated that lacked the enzyme. The mutant could no longer use leucine, isoleucine or valine as sole carbon sources (Hafner *et al.*, 1991). Nothing on the regulation of the enzyme was reported.

A cluster of genes, *bkdABC*, was cloned from *S. avermitilis* (?). The cluster had homology to other bacterial α-keto acid dehydrogenase-encoding genes. When expressed in *E. coli*, the product of these genes had an α-keto acid dehydrogenase activity; however, when disrupted in *S. avermitilis* there was no effect on avermectin production or branched-chain amino acid catabolism (Skinner *et al.*, 1995). A second cluster of α-keto acid dehydrogenase-encoding genes, *bkdFGH*, were identified in this streptomycete 12.5 kb away from *bkdABC*, and disruption of *bkdFGH* did generate a mutant unable to catabolize valine, leucine and isoleucine as sole carbon sources and unable to make avermectin (Denoya *et al.*, 1995). Presumably the Hafner *et al.* (1991) mutant affected *bkdFGH*. The α-keto acid dehydrogenase of *S. ambofaciens* (A23), like the VDH, was valine-induced and ammonium-repressed (Lounes *et al.*, 1995a).

3.3.4.3. Methylmalonic Acid Semialdehyde Dehydrogenase. Methylmalonic acid semialdehyde dehydrogenase is the sixth enzyme in the valine catabolism pathway. The gene encoding this enzyme, *msdA*, was identified in *S. coelicolor* A3(2) by homology to a *Pseudomonas aeruginosa* enzyme. Disruption of *msdA* generated a mutant unable to use valine or isobutyrate (Zhang *et al.*, 1996).

3.3.5. The Aspartate Family of Amino Acids

3.3.5.1. Aspartate. *S. fradiae* (G68) can use aspartate as sole nitrogen source but not as sole carbon source. This is because it possesses glutamate:oxaloacetate transaminase (GOAT) or aspartate aminotransferase, (see section 3.6.4) but not aspartase or aspartate dehydrogenase (Romano and Nickerson, 1958). The enzyme was purified and its kinetic properties assessed. Activity of the enzyme was suppressed by high concentrations of ammonium (Lee and Lee, 1993). It is possible, therefore, for the amino group of aspartate to be assimilated but the oxaloacetate moiety cannot be used in the absence of some other carbon and energy source.

Fisher (1989) reported the presence of GOAT in *S. coelicolor* A3(2) (A21) but did not investigate the presence or absence of other aspartate metabolism enzymes. This streptomycete, like *S. fradiae* (G68), cannot use aspartate as a carbon source (Hodgson, 1980). GOAT was also identified in *S. noursei* (?) (Grafe *et al.*, 1974b).

3.3.5.2. Asparagine. Some of the regulatory properties of asparaginases from *S. venezuelae* (A6) and *S. karnatakensis* (C44) have been reported (Mostafa, 1979). The enzyme catalyses the hydrolysis of asparagine to aspartate and ammonium and can be used in the treatment of human leukaemia. Enzyme yields were reduced when glycerol, glucose, fructose, sucrose, lactose or maltose were substituted for starch. Therefore the enzyme appeared to be subject to carbon catabolite repression. The enzyme was fully induced in both organisms when asparagine was used as the sole nitrogen source. Glutamine could also act as an inducer, albeit less effectively than asparagine. Histidine and aspartate fully repressed this induction, whilst glutamine greatly potentiated it in *S. karnatakensis* (C44). In *S. venezuelae* (A6) aspartate repressed the asparaginase but histidine behaved as an inducer and potentiator of asparagine induction, like glutamine. It should be noted that the medium used for this experiment was not buffered and so, as the author rightly states, the apparent repression of the enzyme by aspartate could be due to pH effects.

3.3.5.3. Threonine. Threonine is a substrate for threonine dehydratase and threonine aldolase. Both enzymes have been identified in *S. fradiae* (G68) (Vancura *et al.*, 1988b). The dehydratase, which catalyses the deamination of

threonine to α-ketobutyrate, has been identified in a number of streptomycetes. It appears that this enzyme has an amphibolic role in the cell: an anabolic role in the synthesis of isoleucine, and a catabolic role in the degradation of threonine. α-Ketobutyrate is a precursor of propionate, which in *S. fradiae* (G68) is a precursor of protylonolide, the polyketide backbone of tylosin (section 3.3.4.1). Threonine induced the enzyme in *S. fradiae* (G68) rather than repressed it, which might imply a catabolic role. This suggestion is supported by the observation that ammonium inhibited the enzyme. Vancura *et al.* (1988b) claimed that the enzyme was repressed by ammonium, but their data did not include a comparison between ammonium grown cells, threonine grown cells, and threonine plus ammonium grown cells. Therefore, the observed reduction of activity in the presence of ammonium alone could be due to lack of induction. Another piece of evidence that supports a purely catabolic role for this enzyme was the observation that cells fed with threonine showed an increase in unbranched fatty acids with an even number of carbon atoms in the chain.

Purification of *S. fradiae* (G68) threonine dehydratase has been achieved (Lee and Lee, 1992) and the K_m for threonine was 21 mM. ATP activated the enzyme and ammonium ions significantly inhibited it. These authors concluded that ammonium 'repression' of tylosin was due to ammonium suppression of aspartate aminotransferase, threonine dehydratase and valine dehydrogenase, all of which supply precursors to tylosin (Lee and Lee, 1992, 1993). The purified enzyme was used to clone the threonine dehydratase gene from *S. fradiae* (G68) (Lee and Lee, 1995).

The threonine dehydratase of *S. clavuligerus* (J71) was neither repressed nor induced by threonine, but was induced 3.3-fold by ammonium (Bascaran *et al.*, 1989a). It is difficult to interpret these data in terms of a purely anabolic or catabolic role for the enzyme.

Threonine aldolase catalyses the splitting of threonine into glycine and acetaldehyde and is a purely catabolic enzyme. It is presumed that acetaldehyde is converted into acetyl CoA. In *S. fradiae* (G68) this enzyme was induced about two-fold by threonine, and this induction appeared to be independent of the presence of ammonium ions or glutamate (Vancura *et al.*, 1988b).

3.3.5.4. Lysine. Lysine catabolism by streptomycetes has been investigated because the amino acid is a precursor of a number of commercially important secondary metabolites; e.g. it is converted into α-aminoadipate, the precursor of β-lactams (Kern *et al.*, 1980). The pathway of lysine catabolism, which released assimilatable nitrogen and carbon, was via cadaverine, in all streptomycetes tested. The cadaverine pathway involves: oxidation of lysine to cadaverine with the concomitant release of CO_2; transamination to 1-piperideine; dehydrogenation to δ-aminovalerate; transamination to glutarate semialdehyde; and, finally, dehydrogenation to glutarate (Fothergill and Guest,

1977). The resulting glutarate is presumed to be catabolized via glutaryl-CoA and the fatty acid degradation pathway to yield two molecules of acetyl CoA. The second enzyme of the cadaverine pathway is cadaverine aminotransferase and this enzyme was found in all streptomycetes tested – i.e. *S. clavuligerus* (J71), *S. glaucescens* (A28), *S. griseus* (A1B), *Nocardia* (*Streptomyces*) *lactamdurans* (A7), *S. lividans* (A21), *S. parvulus* (A12), *S. phaeochromogenes* (A40), *S. rimosus* (B42), *S. venezuelae* (A6) and *S. viridochromogenes* (A27). All of these could use lysine as a nitrogen source. The cadaverine aminotransferase of *S. clavuligerus* (J71) was induced by lysine (3- to 7.3-fold, depending on the medium) but more efficiently by cadaverine. Ammonium and glutamate and the preferred carbon source, glycerol, repressed this induction (Madduri *et al.*, 1989, 1991).

Only β-lactam-producing streptomycetes, *S. clavuligerus* (J71), *Nocardia* (*Streptomyces*) *lactamdurans* (A7) and *S. griseus* (A1B), also contain lysine ε-aminotransferase (LAT), the first enzyme of the α-aminoadipate degradation pathway. However, this enzyme was exclusively involved in β-lactam biosynthesis as: (1) none of these streptomycetes could use α-aminoadipate as nitrogen source; (2) *S. clavuligerus* (J71) mutants that had lost genes in the cadaverine pathway could not catabolize lysine (Madduri *et al.*, 1989); (3) *S. clavuligerus* (J71) mutants that had lost LAT could still catabolize lysine but could not produce β-lactams (Romero *et al.*, 1988); (4) *S. clavuligerus* (J71) LAT was regulated by the same metabolites as regulated β-lactam production – i.e. carbon catabolite 'repression' and lack of lysine induction (Madduri *et al.*, 1991) Rius *et al.* (1996) later reported that LAT could be induced with high concentrations of lysine; and (5) the gene encoding the enzyme was part of the β-lactam biosynthetic gene cluster (Tobin *et al.*, 1991). There is evidence that a direct or indirect product of LAT action is the inducer of β-lactam biosynthesis as a LAT mutant was unable to induce the genes encoding ACV synthetase and isopenicillin *N*-synthetase (Yu *et al.*, 1994).

Surprisingly, *S. lividans* (A21), *S. glaucescens* (A28) and *S. venezuelae* (A6), which were unable to produce β-lactams, could utilize α-aminoadipate, even though they could not synthesize it from lysine. Madduri *et al.* (1989) did not report what effect, if any, the loss of the cadaverine pathway had on β-lactam production.

Nikkomycin is a chitin synthesis inhibitor that is believed to be derived from piperideine-6-carboxylate, an intermediate in the α-aminoadipate pathway of lysine catabolism. The nikkomycin producer, *S. tendae* (A12), could also use L-lysine but not D-lysine as sole nitrogen source. However, there was no evidence of the presence of lysine decarboxylase, the cadaverine pathway enzyme, whilst lysine α-transaminase was present. Mutants unable to utilize lysine as a nitrogen source were still able to produce nikkomycin. In all cases they retained the lysine α-transaminase activity (Jordan *et al.*, 1985). It is possible that this streptomycete contains the cadaverine pathway but that the

decarboxylase enzyme was too unstable to be assayed and that the lysine α-transaminase was involved in secondary metabolism.

S. pilosus (A37) produces the siderophore desferrioxamine B. This iron-chelating compound was synthesized from lysine following decarboxylation of the amino acid to cadaverine. The synthesis of the enzyme lysine decarboxylase was shown to be regulated by iron. In low-iron media, synthesis increased as the culture entered late exponential phase. It was not clear whether this enzyme was induced by lysine or whether it had a role in lysine catabolism. Mutants that lacked the enzyme were isolated but their effect, if any, on lysine catabolism was not reported (Schupp *et al.*, 1987).

3.3.6. The Pyruvate Family of Amino Acids

Leucine catabolism and valine catabolism have been discussed above (section 3.3.4). Alanine is a special case as it is simply synthesized by the amination of pyruvate and catabolized by the reverse reaction. It is therefore often impossible to say whether any particular enzyme is anabolic or catabolic, so the process is classified as amphibolic. Amination of pyruvate or deamination of alanine can occur by either transamination (e.g. alanine:2-oxoglutarate transaminase; AOAT) or by condensation of pyruvate with ammonium with the consumption of reducing power via the back reaction of alanine dehydrogenase (ADH). By studying the regulation of AOAT and ADH, we may be able to assess whether they are anabolic or catabolic.

AOAT activity could be found in neither *S. coelicolor* A3(2) (A21) (Fisher, 1989) nor *S. clavuligerus* (J71) (Brana *et al.*, 1986a). However, it was found in *S. hygroscopicus* (A32) and *S. noursei* (?) (Grafe *et al.*, 1974a, b), *S. cyanogenus* (?) (Watanabe *et al.*, 1976a) and *S. avermitilis* (?) (Novak *et al.*, 1992a). Alanine:2-isovalerate transaminase could not be found in *S. coelicolor* A3(2) (A21) (Fisher, 1989). This raises the possibility that alanine catabolism and biosynthesis may be possible by transamination in *S. hygroscopicus* (A32) and *S. noursei* (?), *S. cyanogenus* (?) and *S. avermitilis* (?).

In contrast to the transaminases, ADH activity was found in all streptomycetes examined (Aharonowitz and Friedrich, 1980; Redman and Hornemann, 1980; Shapiro and Vining, 1980; Itoh and Morikawa, 1983; Vancurova *et al.*, 1988a; Vancura *et al.*, 1989b). Therefore it is assumed that, in most cases, alanine synthesis and catabolism is carried out by ADH. The observation of alanine induction of the enzyme implies a catabolic role, whereas ammonium induction of the enzyme implies an anabolic role.

ADH has been purified from *S. aureofaciens* (A14) (Vancurova *et al.*, 1988a; Nguyen *et al.*, 1995g), *S. clavuligerus* (J71) (Aharonowitz and Friedrich, 1980), *S. fradiae* (G68) (Vancura *et al.*, 1989b) and *S. phaeochromogenes* (A40) (Itoh and Morikawa, 1983). There was a lot of variation in subunit size and

number between the enzymes. The affinities for the various substrates are of the same order except ammonium, where the K_m varied from 6.67 mM (*S. aureofaciens*) to 61.0 mM (*S. phaeochromogenes*). It has been reported that *S. venezuelae* (A6) ADH has a K_m for ammonium ions of 184 mM (Shapiro and Vining, 1983). There was a great deal of variability in substrate specificity, with the *S. aureofaciens* (A14) enzyme being most restricted.

Ammonium and alanine induced AOAT and ADH activity in *S. avermitilis*. AOAT was induced only three- to four-fold by both compounds, whereas ADH was induced five-fold by the salt and 86-fold by the amino acid (Novak *et al.*, 1997). Ammonium and alanine induced ADH activity in *S. hygroscopicus* (A32) and *S. noursei* (?). ADH activity was also increased in *S. hygroscopicus* (A32) as the culture entered the stationary phase (Grafe *et al.*, 1974a, 1979). Three reports concerning *S. clavuligerus* (J71) ADH are available (Aharonowitz and Friedrich, 1980; Brana *et al.*, 1986a, b) which are contradictory in detail. All agree that ADH is induced by alanine but the exact level varied from 25- to 73-fold, using the ADH level in arginine as base level. Ammonium was also an effective inducer, 20- to 31-fold, and potentiated the induction 1.15- to 1.3-fold by alanine. Ammonium also led to the induction of the enzyme by greater than ten-fold in the presence of amino acids, which were not inducers in themselves, with the exception of glutamate, and the results were contradictory with glutamine. The two Brana *et al.* papers report histidine as an inducer (about six-fold) whilst Aharonowitz and Friedrich (1980) reported no induction with this amino acid.

Itoh and Morikawa (1983) reported that the ADH of *S. phaeochromogenes* (A40) was induced 78.5-fold by L-alanine and 27-fold by D-alanine. A D-alanine racemase could not be found, which implied that the induction system was not stereospecific. A similar observation was made for *Saccharopolyspora erythraea* (previously *S. erythreus* (A1C)) ADH (Roszkowski *et al.*, 1969). The other inducer identified in *S. phaeochromogenes* (A40) was D,L-glutamate and glucose was a repressor, which implied a catabolic role of the enzyme. Fisher (1989) reported that alanine induced the activity of ADH by some 100-fold in *S. coelicolor* A3(2) (A21). Nothing was reported on the ability of ammonium to repress or potentiate this induction. Ammonium alone led to a very low-level induction of the enzyme (1.75-fold).

3.3.7. The Serine Family of Amino Acids

Nothing has been reported on the catabolism of glycine in streptomycetes. The enzyme of catabolism of serine, serine dehydratase (or deaminase), has been reported in a naturally serine-sensitive strain of *S. aureofaciens* (A14) (Parada, 1981). The enzyme was not affected in a serine-resistant mutant, but otherwise there was no mention of the control of the enzyme. Serine dehydratase (deaminase) activity was also found in cells of *S. clavuligerus* (J71) cultivated in

threonine or ammonium as nitrogen source (Bascaran *et al.*, 1989a). However, the streptomycete could not grow on serine as the sole nitrogen source. Ammonium grown cells had 1.4-fold more enzyme than did threonine grown cells. Threonine plus ammonium grown cells had 2.8-fold more enzyme than threonine grown cells. The inability to grow on serine and the stimulation by ammonium might imply that the enzyme was not involved in catabolism. An alternative role might be the detoxification of serine. Serine toxicity was observed in *S. rimosus* (B42) as well as in *S. aureofaciens* (A14).

3.3.8. The Sulphur-Containing Amino Acids

Little or nothing has been reported on cysteine catabolism in streptomycetes. The *trans*-sulphuration pathway (see section 4.1.8.2.) could be considered a methionine catabolic pathway, in a sense. Hagino and Nakayama (1968) reported the accumulation of 3-methylthiopropylamine, the methionine decarboxylation product, by *Streptomyces* sp. K37 when it was grown in the presence of methionine. The enzyme responsible was found to be inducible by methionine and to be methionine-specific (K_m = 0.0121 mM). The physiological role of the enzyme was unclear. Stevenson *et al.* (1990) reported the purification and a detailed study of the kinetic properties of the methionine decarboxylase from an unclassified *Streptomyces* species.

3.4. Nucleotide Catabolism

3.4.1. Pyrimidines

Thymine was shown to be catabolized via β-aminoisobutyrate in *S. cinnamonensis* (?) as radiolabelled thymine led to radiolabelled monensin of the same type if radiolabelled β-aminoisobutyrate was added. The presumed pathway of catabolism of the nucleotide is thought to be reduction to dihydrothymine, ring breakage to β-ureidoisobutyric acid, cleavage to urea and β-aminoisobutyrate, deamination to methylmalonyl semialdehyde, condensation with cofactor A to methylmalonyl CoA and rearrangement to succinyl-CoA, thence to the TCA cycle (section 2.1.2) (O'Hagan *et al.*, 1995).

3.4.2. Purines

Watanabe, Ohe and coworkers have published a substantial body of work dedicated to the understanding of the mechanism and regulation of purine catabolism and the purine salvage pathway. The salvage pathway allows the

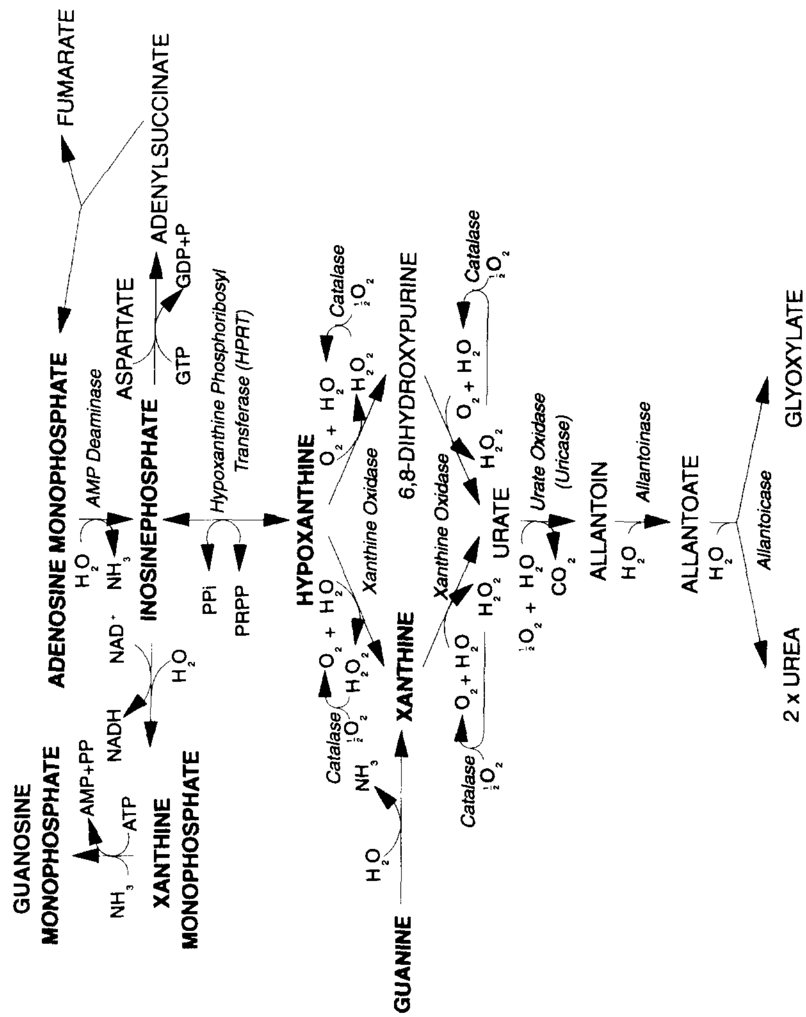


Figure. 2 Purine catabolism in streptomycetes (see text for references).

interconversion of purines – i.e. hypoxanthine, inosine monophosphate, AMP and GMP. The streptomycete used was a strong uricase producer, originally isolated from the soil of a poultry farm (Watanabe *et al.*, 1969) and eventually identified as *S. cyanogenus* (?) (Ohe and Watanabe, 1979). The purine catabolism and salvage pathway of streptomycetes is presented in Fig. 2. Hypoxanthine acted as sole nitrogen source for *S. cyanogenus* (?) and so presumably allantoinase and allantoicase were present, but they were not assayed (Ohe and Watanabe, 1977). Urea must be converted to ammonium via urease (see section 3.5.3).

There are parallel pathways of hypoxanthine oxidation to urate in streptomycetes. When incubated with hypoxanthine a number of *Streptomyces* species accumulated xanthine, the more usual intermediate, and 6,8-dihydroxypurine (DHOP). Both of these intermediates were further catabolized to urate. In the presence of chloramphenicol, xanthine was catabolized to urate but only DHOP accumulated. This implied that the enzyme responsible for oxidation of DHOP was different from the xanthine oxidase/dehydrogenase (XDH) that carried out the hypoxanthine and xanthine oxidation steps (Watanabe and Ohe, 1972). However, when the properties of purified XDH were examined it was found that this enzyme oxidized DHOP at 52% of the rate of oxidation of xanthine (Ohe and Watanabe, 1979). It would appear, therefore, that the DHOP pathway is a side reaction of XDH catalysed oxidation of hypoxanthine.

Four of the enzymes of the pathway have been purified: hypoxanthine phosphoribosyl-transferase (HPRT) (Ohe and Watanabe, 1980); xanthine dehydrogenase (XDH) (Ohe and Watanabe, 1979); uricase (Ohe and Watanabe, 1981); and adenine deaminase (Jun *et al.*, 1991). The XDH had some unusual properties in that no molybdenum could be detected in the purified enzyme. The enzyme had a higher affinity and reaction rate with hypoxanthine than with xanthine, which was unexpected. In addition, guanine had the highest affinity of all the substrates tested, although the reaction rate was a little slower than with hypoxanthine. The product of the guanine reaction was 8-hydroxyguanine; however, the fate of this compound in the cell was not investigated further. Uricase (urate oxidase) was also unusual in that there was no evidence of copper, iron, manganese or flavin being present in meaningful stoichiometric amounts.

The regulation of the pathway was complex. This probably reflects its amphibolic (catabolic and anabolic) nature. HPRT appeared to be constitutive and, in exponentially growing cells supplied with hypoxanthine, the salvage pathway of IMP synthesis appeared to predominate. However, as the cells entered the late exponential phase, XDH was induced and oxidation of hypoxanthine and IMP synthesis occurred concurrently. In nitrogen-starved cells HPRT was present and XDH activity was induced, particularly if hypoxanthine was present. IMP synthesis was curtailed and catabolism of hypoxanthine

then predominated (Watanabe *et al.*, 1976b). There might be a mechanism to decrease IMP synthesis as HPRT has a higher affinity, (K_m = 1.6 μM), for hypoxanthine than does XDH (K_m = 55 μM).

The catabolic form of the pathway appeared to release ammonium for nitrogen assimilation, a suggestion supported by the observations that: (1) hypoxanthine could be used as the sole nitrogen source; (2) xanthine, hypoxanthine and other purines induced both XDH and uricase; and (3) ammonium in the growth medium repressed induction of the enzymes. The induction of XDH and uricase was inhibited by the presence of chloramphenicol, which demonstrated it was true repression (Watanabe and Fukumoto, 1970; Watanabe *et al.*, 1969, 1976a; Ohe and Watanabe, 1977, 1978). In earlier papers the authors seemed to assume that urate was the true inducer of uricase. However, other purines, particularly hypoxanthine, were more efficient inducers. Conditions were found in which urate would induce uricase, but this required the use of resting (non-growing) cells and, indeed, they found that provided glucose was present, not even urate was needed (Watanabe and Fukumoto, 1970; Watanabe, 1971; Watanabe and Ohe, 1973; Watanabe *et al.*, 1973). A simpler explanation would be that hypoxanthine was the inducer of XDH and uricase and that the conditions in which urate induced uricase (i.e. severe nitrogen starvation in the presence of a carbon source) were conditions in which the true inducer, hypoxanthine, was generated from urate by low-level XDH activity.

Presumably allantoinase and allantoicase were also induced by hypoxanthine, because 'urate induced' uricase converted equimolar amounts of urate to allantoin (Watanabe and Fukumoto, 1970). Urate appeared to be transported by a facilitated diffusion mechanism with a K_m of 500 mM. Xanthine alone, among a range of purines tested, competed for the urate transporter (Watanabe *et al.*, 1972).

There seemed to be a complex interplay of glucose and ammonium regulation of both XDH and uricase. As noted above, glucose in the absence of a nitrogen source did not repress induction of uricase; rather it promoted its formation in the absence of purines. The same was true of XDH. Glucose and ammonium together repressed both enzymes at the level of transcription. Glycerol could be substituted for glucose and a wide variety of amino acids could be substituted for ammonium. In fact, glutamate, glutamine, aspartate and asparagine caused greater repression than ammonium. Carbon limitation released nitrogen catabolite repression and nitrogen limitation released carbon catabolite repression. Cyclic nucleotides appeared to play no role in carbon catabolite repression (Watanabe *et al.*, 1973, 1976a; Ohe and Watanabe, 1977, 1978).

Activity of an extracellular adenosine deaminase, another enzyme of the purine salvage/catabolism pathway, was shown to be present during the growth phase but to decrease during the stationary phase in an uncharacterized

streptomycete (Jun *et al.*, 1991). The medium used was complex, containing peptone and meat extract, and it was not clear whether adenine was present in great quantity. It is impossible to say, therefore, whether the enzyme was adenine induced or not.

3.5. The Mineral Origins of Ammonium

As in other bacteria, glutamine and glutamate are important intermediates of ammonium assimilation in streptomycetes. Before we discuss ammonium assimilation let us discuss the origin of ammonium in cells. One major source of ammonium within the cell is from the catabolism of amino acids (section 3.3).

3.5.1. Nitrogen Fixation

A number of reports claim that streptomycetes fix atmospheric nitrogen. These reports usually rely on the ability of the strains to grow on media unsupplemented with a fixed nitrogen source. However, when nitrogenase, the crucial enzyme of nitrogen reduction, was sought, using the acetylene reduction assay, none could be found (Pearson *et al.*, 1982). It might be supposed that this ability reflects cryptic growth and/or the ability to scavenge fixed nitrogen contaminants within the growth media or from the atmosphere; i.e. it was equivalent to oligocarbophily (section 1.4). However, a recent report on the ability of *S. thermoautotrophicus* (?) to fix $^{15}N_2$ again raises the question of nitrogen fixation in other streptomycetes as the *S. thermoautotrophicus* mechanism of nitrogen fixation was incapable of acetylene reduction (Ribbe *et al.*, 1997) (section 1.4).

3.5.2. Nitrate Reduction

Nitrate is a useful source of nitrogen for streptomycetes. The presence of nitrate reductase has been documented in a number of strains. Mansour and Shady (1984) assigned streptomycetes to four groups based on nitrate reduction capacity: very weak to weak, *S. cellulosae* (A13) and *S. coerulatus* (?); moderate, *S. flavovirens* (A1C), *S. malachiticus* (A12), *S. roseolilacinus* (G68) and *S. viridosporus* (A15); active, *S. longisporus* (A18) and *S. galilaeus* (A19); and most active, *S. fulvoviridis* (A3) and *S. roseofulvus* (A14). These authors also reported the effect of media constituents on the *S. fulvoviridis* (A3) enzyme. Glucose was the best carbon source for production, but sorbose and sucrose repressed its production completely. The nature

of the nitrogen source had only a mild effect on the enzyme. Sodium nitrate-grown cells had twice the activity of ammonium sulphate- or chloride-grown cells.

Shapiro and Vining (1984) reported that *S. venezuelae* (A6) showed diauxic growth on medium containing both ammonium and nitrate. The ammonium was catabolized first. Only upon ammonium depletion was nitrate catabolized. Ammonium and nitrate were taken up in preference to proline. However, proline uptake began before either ammonium or nitrate depletion of the medium. Surprisingly, nitrate reductase was a constitutive enzyme that was not inhibited by ammonium, glutamine, alanine or proline. There did appear to be a mild, approximately 33%, inhibition by glutamate. The authors concluded that uptake of nitrate was repressed by ammonium.

Nothing has been reported concerning the regulation of nitrate reduction in *S. coelicolor* A3(2) (A21); but Ortali *et al.* (1980) investigated the genetics of the system. Two classes of mutant were found: those unable to assimilate nitrate and nitrite (*ammA*); and those unable to assimilate nitrate but able to assimilate nitrite (*ammB*). The former has the phenotype expected of nitrite reductase or control mutants, the latter that expected of nitrate reductase mutants. Both classes of mutation mapped to a single locus.

Using a partially purified nitrate reductase preparation from *S. cyanoviridis* (?), Ragab *et al.* (1983) reported that cyanide, azide, 8-hydroxyquinoline, urea, L-histidine and L-asparagine inhibited enzyme activity. The inhibition by the latter three compounds is particularly interesting when considering the role of nitrate reductase in general nitrogen metabolism. These authors reported that a number of amino acids inhibited the enzyme but that histidine and asparagine had the most marked effect.

As well as being a source of ammonium, nitrate and nitrite can also be used instead of oxygen as terminal acceptors of electrons, thus allowing anaerobic respiration (section 2.1.4). In this case the products are dinitrogen (N_2) or nitrous oxide (N_2O), rather than ammonium, and the process is termed 'denitrification'. Albrecht *et al.* (1997) screened 42 streptomycetes and identified five that produced N_2O on nitrate-containing medium. In all cases the streptomycetes could not grow anaerobically in the presence of nitrate. Two denitrifiers, *S. violaceoruber* (A21) and *S. nitrosporeus* (A1C), were studied in which nitrate was converted to N_2O and nitric oxide, NO. In another survey including other actinomycetes, it was demonstrated that of eleven *Streptomyces* spp. tested, all could denitrify nitrate to N_2O to some extent. Under anaerobic conditions, *S. thioluteus* (?), *S. coelicolor* (A1A), *S. lavendulae* (F61), *S. cavourensis* (A1B) and *S. zelensis* (?) showed high levels of nitrate denitrification; *S. lavendulae* (F61), *S. thioluteus* (?), *S. akiyoshiensis* (?), *S. glaucus* (?) and *S. flavotricini* (F61) showed high levels of nitrite denitrification. All the others could be induced to high levels of denitrification under O_2-limited conditions. The membrane-bound nitrite reductase and azurin, a protein involved

in electron donation to the reductases, were purified from *S. thioluteus* (?) (Shoun *et al.*, 1998).

3.5.3. Urease

This enzyme, which catalyses the hydrolysis of urea to ammonium and carbon dioxide, has been studied in a number of streptomycetes because it is easy to assay and shows evidence of being regulated by global nitrogen regulatory mechanisms. Bascaran *et al.* (1989a) reported that, in *S. clavuligerus* (J71), urease activity and glutamine synthetase (GS) activity were suppressed in conditions of high ammonium supply but elevated in conditions of low ammonium supply. Whilst urea stimulated urease activity, the enzyme was present when no urea was included in the growth medium, the implication being that the enzyme was not induced by its substrate. It is possible that urea generated within the cell could have been acting as inducer in conditions where it was not present in the culture medium.

A number of mutants that had lost ammonium suppression of GS also lost ammonium suppression of urease. In addition, mutants that could not express GS activity lost the ability to express urease. However, there were mutants in which the co-regulation of GS and urease had been separated (Bascaran *et al.*, 1989a, b) (see section 3.7.2.2). The nature of the suppression of urease activity by ammonium has not been characterized. It is not yet clear whether it is at the level of gene activity or at the level of enzyme activity. The regulation of GS activity has been shown to occur at both levels (see section 3.6.1). A similar system of coordinate regulation of urease and GS has been discovered in *S. coelicolor* A3(2), and again mutants affecting the ammonium regulation of both enzymes have been identified (Fisher, 1988; Fisher *et al.*, 1990).

3.6. Ammonium Assimilation

The potential pathways of ammonium assimilation in streptomycetes are shown in Fig. 3. The central pathway of ammonium incorporation in bacteria involves the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT). Ammonium is incorporated via the high-affinity enzyme GS, with the expenditure of energy, in the form of ATP. Glutamate is generated by GOGAT by the transamination of α-ketoglutarate (2-oxoglutarate) with glutamine and the expenditure of reduced nicotinamide.

A large number of bacteria have an additional enzyme that can lead to assimilation of ammonium. This enzyme, glutamate dehydrogenase (GDH), has low affinity for ammonium; but at high ammonium concentration it can

catalyse the formation of glutamate directly from ammonium and α-ketoglutarate without the cleavage of ATP. Therefore, the cell can generate both glutamate and glutamine with the GDH–GOGAT couple in high-ammonium conditions, or with the GS–GOGAT couple in low-ammonium conditions.

Not all streptomycetes have GDH; alternatives include the incorporation of ammonium into pyruvate to form alanine, again expending reducing power, by the enzyme alanine dehydrogenase. Here an alanine transaminase has to be present to allow transfer of the amino group to glutamate and thence to glutamine via GOGAT. One such alanine transaminase is AOAT. A second alternative mechanism of ammonium assimilation might be via aspartate. Amination of an organic acid to form aspartate must be followed by transamination of glutamate via glutamate:oxaloacetate transaminase (GOAT), alternatively called aspartate:2-oxoglutarate amino-transferase or aspartate transaminase (Fig. 3).

3.6.1. Streptomycete Glutamine Synthetases

Glutamine synthetase is regulated in enteric bacteria, both at the gene expression level and the enzyme level, by the availability of ammonium. High ammonium concentration represses enzyme synthesis and leads to the inactivation of active enzyme by adenylation. This system of enzyme adenylation appeared to be restricted to Gram-negative bacteria as the GSs of bacilli, clostridia and lactic acid bacteria are not adenylated (Shapiro and Stadtman, 1970). The first report on GS in streptomycetes was that of Tronick *et al.* (1973). These authors examined antigenic cross-reactivity and GS activity in a

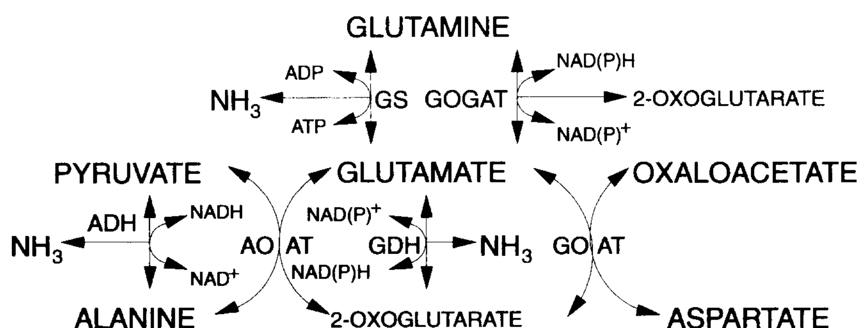


Figure 3 Potential pathways of ammonium assimilation in streptomycetes. GS = glutamine synthetase; GOGAT = glutamate synthase; GDH = glutamate dehydrogenase; ADH = alanine dehydrogenase; AOAT = alanine:2-oxoglutarate (α-ketoglutarate) transaminase; GOAT = glutamate:oxaloacetate transaminase (see text for references).

wide range of bacteria, including two streptomycetes, *S. rutgersensis* (A1A) and *S. diastatochromogenes* (A19). No adenylation of the streptomycete GSs was found, but the streptomycete enzymes showed immunogenic cross-reactivity with GSs of enteric bacteria but not those of other Gram-positive bacteria. This was the exception to the rule that adenylatable GSs showed cross-reactivity only with like enzymes and not with non-adenylatable enzymes.

The GS of *S. cattleya* (C47) has been studied extensively. GS activity was rapidly inactivated by ammonium shock (Wax *et al.*, 1982) and the molecular basis of this inactivation was the adenylation of the enzyme (Streicher and Tyler, 1981). The apparent contradiction with the previous observations was resolved because the *S. cattleya* enzyme was not adenylated under the conditions used by Tronick *et al.* (1973). Sequence analysis of the *S. coelicolor* A3(2) (A21) GS gene by Wray and Fisher (1988) demonstrated that there was greater similarity between the GSs of streptomycetes and enteric bacteria than between the GSs of streptomycetes and bacilli. Therefore, in terms of adenylation, immunological cross-reactivity and protein sequence homology, streptomycete GS is more like enteric bacteria GS than the GSs of other Gram-positive bacteria. The *S. cattleya* enzyme also has a subunit of the same molecular weight as the *E. coli* enzyme (Paress and Streicher, 1985).

Differences were observed between *S. cattleya* (C47) GS and GSs from enteric bacteria. In enteric bacteria adenylation abolished the forward reaction of the enzyme, but not the reverse reaction (i.e. the γ-glutamyl transferase activity), whilst adenylation of the streptomycete enzyme abolished both activities (Wax *et al.* 1982). Streicher and Tyler (1981) reported that the streptomycete enzyme was *not* inactivated by magnesium ion, unlike the enzyme from enteric bacteria, *S. venezuelae* (A6) (Shapiro and Vining, 1983) and *S. clavuligerus* (J71) (Brana *et al.*, 1985). Paress and Streicher (1985) reported that the enzyme responsible for adenylation of *S. cattleya* GS could not adenylate *E. coli* GS, nor could the *E. coli* enzyme adenylate the streptomycete enzyme.

Streicher and Tyler (1981) had reported that the levels of GS protein within cells grown under different conditions could vary as much as 20-fold. Later work revealed that growth on ammonium salts repressed GS gene expression by eight-fold when compared with cells grown on glutamate (Paress and Streicher, 1985). GS gene expression appeared to be independent of the growth phase and the generation time. The steady-state level of adenylation of the GS in cells never exceeded 16% of the total even under the most repressive conditions; i.e. NH_4Cl plus glutamate as nitrogen source. When NH_4Cl was added to cells that were growing under conditions of high GS level, GS activity was inactivated by adenylation. Secondly, *de novo* synthesis of the enzyme ceased until GS concentration within the cell had decreased to that seen in cells that had been growing continuously on

NH_4Cl. The dilution of the enzyme to new steady-state levels took 6.5 hours.

Fisher and Wray (1989) demonstrated that the GS of *S. coelicolor* A3(2) (A21) was regulated in the same manner as that of *S. cattleya* (C47); i.e. at the level of gene expression and by enzyme adenylation. In this strain, however, it was possible to find conditions where 50% of the enzyme was adenylated. The conditions included growth on glutamine plus aspartate as sole nitrogen sources. During growth in complex medium, expression of the GS gene was repressed six-fold when compared with non-repressing conditions (i.e. growth with histidine as the sole nitrogen source). Quantitative primer extension analysis demonstrated that repression of GS gene expression was transcriptional. Sequence analysis of the GS gene (*glnA*) showed it was monocistronic, unlike GS genes in other bacteria, where more usually the *glnA* gene is associated with the global nitrogen regulatory genes *ntrB* and *ntrC*.

Therefore, regulation at the gene and enzyme levels is important in the steady-state regulation of GS activity. The level of adenylation of GS was much higher in *glt5* and *glt11* mutants deficient in GOGAT activity. This led to the suggestion that glutamine, or a metabolite, is the regulator of GS adenylation. GS gene expression was not affected in these mutants. This illustrates a clear difference with enteric bacteria where GS gene expression and GS adenylation are coordinately regulated. In *S. coelicolor* A3(2) (A2l) it was possible to find conditions where GS gene expression was completely derepressed yet GS adenylation still occurred. There was no evidence of glucose repression of the enzyme.

The regulation of GS activity has been reported in *S. clavuligerus* (J71) (Brana *et al.*, 1986a). Again, regulation occurred at the level of gene expression and enzyme inactivation. Bascaran *et al.* (1989a) showed that the mechanism of enzyme inactivation was enzyme adenylation. The modulation of activity of GS activity in *S. noursei* (?) was reported by Grafe *et al.* (1977). Enzyme activity was low in media containing ammonium, but when the ion was exhausted the activity of GS increased. If, however, the medium contained amino acids in addition to ammonium, then GS activity remained high. The degree of stimulation depended on the amino acid present. Aspartate had the greatest effect, followed by alanine (60-fold) < glycine (×46) < serine (×28) < glutamate (×18) < isoleucine (×8) < lysine (×7) < proline (×4) < glutamine (×2.5). The authors postulated that there could be two forms of GS in the cell, each under different regulatory control; however, they made no speculation regarding the mechanism of GS control.

The GS of *S. venezuelae* (A6) appears to be regulated in a different manner to all the streptomycete GS enzymes discussed so far. As in other streptomycetes, ammonium shock caused a decrease in GS activity. However, GS activity could not be recovered by incubation with snake venom phosphodiesterase, which removed the adenyl group from other GSs (Shapiro and Vining,

1983). Also, the steady-state levels of GS activity did not appear to be affected by growth rate or nitrogen source depletion.

Xia and Jiao (1986) reported some of the properties of GS purified from *S. hygroscopicus* var. *jinggangensis* (A32). The enzyme was made up of 12 identical subunits of 43 000 M_r, which were arranged in two hexagonal rings, one surmounting the other in a face-to-face formation. This is the same structure as found in enteric bacteria GS. Kumada *et al.* (1990b) reported the subunit size of *S. hygroscopicus* SFI293 GS was 55 000 M_r. Inhibition studies of the *jinggangensis* enzyme revealed that glutamine and a number of other amino acids gave minor inhibition, whilst AMP and ADP inhibited between 67% and 93% of the control enzyme activity, depending on the metal ion present. In all cases the inhibitors were present at 10 mM concentration, which seems excessive compared with physiological conditions. There was also evidence of cumulative feedback inhibition but, again, the results were not striking. What was striking was the inactivation of GS by the addition of ammonium to the cell culture. Evidence was presented that this inactivation was the result of enzyme adenylation. There was also indirect evidence that ammonium repressed GS formation.

All of the above requires re-examination following discovery that many streptomycetes contain two forms of GS, which had been presaged by the work of Grafe *et al.* (1977) (see above). The discovery was made concurrently by two groups investigating the production of the GS inhibitor phosphinothricyl-alanyl-alanine or bialaphos. Each group was investigating the nature of the resistance determinant in their respective producing strains. Behrmann *et al.* (1990) isolated a gene from *S. viridochromogenes* (A27) that gave rise to resistance when present on a high copy number vector. This determinant proved to be homologous to the GS of eukaryotes and the GSII of *Rhizobium* spp. Using the *glnA* gene of *S. coelicolor* A3(2) and their *glnII* gene, they probed a range of other streptomycetes. Both genes were present in all strains tested – *S. viridochromogenes* (A27), *S. coelicolor* A3(2) (A21), *S. cattleya* (C47), *S. parvulus* (A12), *S. lividans* (A21), *S. galilaeus* (A19), and *S. griseus* (A1B). Streptomycete cells bearing *glnII* on a plasmid had increased levels of GS activity, which, like the GS activity of eukaryotes and *Rhizobiaceae* GSII, proved to be thermolabile. When total GS activity of wild-type *S. viridochromogenes* (A27) was assayed, it was found that 25% of the activity was thermolabile, implying that this alternative GS, GSII, had a role in the cells' nitrogen catabolism. Inactivation of *S. viridochromogenes* (A27) *glnA* or *glnII* by insertional mutation had little affect on the ability of the mutant to grow on low levels of ammonium. However, inactivation of both genes in the same strain abolished this ability. In wild-type cells, GSI (*glnA* product) was the predominant activity and GSI but not GSII was inactivated by adenylation as described above. Amino acids and nucleotides inhibited both GSI and GSII enzyme activities (Hillemann *et al.*, 1993).

Kumada *et al.* (1990a) identified a gene from the bialaphos producer, *S. hygroscopicus* SFI293 (A32), that could complement an *E. coli glnA* mutation. The gene was not expressed from its own promoter in *E. coli*. When transferred to *S. lividans* (A21) on multicopy vectors it was found to endow resistance to bialaphos. The gene was expressed to high levels in these circumstances. The product was purified and shown to be a GS activity (GSII) that was different from the bialaphos-sensitive GSI activity previously identified from *S. hygroscopicus* SFI293 (A32) (Kumada *et al.*, 1990b). GSII activity was thermolabile, and sequencing of the gene revealed that homology to the eukaryotic and *Rhizobium* spp. GSII genes. This gene was designated as *glnB* and was used as a homology probe to show that all streptomycetes tested – S. *glaucescens* (A28), *S. fradiae* (G68), *S. coelicolor* A3(2) (A21), *S. lividans* (A21), *S. viridochromogenes* (A27) and *S. hygroscopicus* (A32) – contained *glnB* and *glnA* analogues. Rochefort and Benson (1990) reported that *Frankia* sp. CpI1, an actinomycete nitrogen-fixing plant symbiont, also contained a GSII gene.

GSI has been purified and characterized from *S. aureofaciens* (A14) (Nguyen *et al.*, 1995e, f) and *S. cinnamonensis* (?). The enzyme from the latter proved to be unusually heat-labile (Nguyen *et al.*, 1997b).

The observation of two GSs in streptomycetes explains the inability of Fisher to isolate a structural GS mutant of *S. coelicolor* A3(2) (A21). Such a mutant would require the inactivation of two genes. By analogy with the eukaryotic GS studies and from examination of the protein sequence, it is to be expected that GSII activity cannot be inactivated by adenylation; which explains why complete inactivation was never found *in vivo* in those streptomycetes tested and found to contain adenylatable GS activities. However, conditions could not be found which allowed any expression of the GSII enzyme, and starvation for nitrogen led to a 56.8-fold derepression of the *glnA* (GSI) gene (Nguyen *et al.*, 1994). Nevertheless, disruption of the *glnA* gene of this streptomycete did not lead to glutamine auxotrophy (Fink *et al.*, 1999).

The *glnE* gene has recently been identified in *S. coelicolor* A3(2) (A21) using consensus sequences from other *glnE* genes. The gene encodes an adenyltransferase that inactivates GSI during ammonium shock and was found to map close to *glnA* and *glnII*. Inactivation of *glnE* led to a GSI that was insensitive to ammonium shock, but there was only 60% of the wild-type GSI activity in the *glnE* mutant, possibly implying a regulatory role for the enzyme. GSI activity that had been lost following ammonium shock could be recovered using snake venom phosphodiesterase (Fink *et al.*, 1999).

A number of nitrogen-deregulated mutants were isolated in *S. clavuligerus* (J71) that had lost ammonium repression of nitrogen catabolism systems. Interestingly seven of the nine mutants identified now contained a thermosensitive GS activity (Bascaran *et al.*, 1989b). These mutants had lost the GSI activity but retained the naturally thermolabile GSII activity. It is not yet

known how GSII activity is regulated in streptomycetes and whether it is co-ordinated with GSI activity.

3.6.2. Streptomycete Glutamate Synthases

GOGAT has been identified in all streptomycetes so far analysed. In *S. noursei* (?) the enzyme had a specific requirement for NADH. No evidence could be found of any coordinate regulation of GOGAT and GS or GDH within this strain (Grafe *et al.*, 1977). Brana *et al.* (1986a) reported that the GOGAT of *S. clavuligerus* (J71) required NADH as a cofactor, and the activity levels appeared to be independent of the nitrogen source in the culture media.

The activity of the NADH-dependent GOGAT in *S. coelicolor* A3(2) (A21) was found to be modulated by the nitrogen source of the cell. When alanine was the nitrogen source GOGAT activity was almost seven-fold lower than when the cell was growing on a complex nitrogen source (Fisher, 1989). Therefore, GS and GOGAT activities are modulated by the nitrogen source in this strain. Xia and Jiao (1986) observed a similar system in *S. hygroscopicus* (A32). They found that both GS and GOGAT activities were reduced in media containing ammonium. The observation that GOGAT activity was reduced by ammonium is the opposite of what was observed in *S. coelicolor* A3(2) (A21).

In *S. venezuelae* (A6), the NADH-dependent GOGAT also appeared to be regulated by the concentration of the nitrogen source present in the culture medium (Shapiro and Vining, 1983). High ammonium concentration in the medium stimulated both GOGAT and GDH, whilst 'poor' nitrogen sources repressed the activity of both these enzymes. As discussed above, in this streptomycete steady-state levels of GS did not appear to be regulated by a nitrogen source. In *S. venezuelae* (A6), therefore, GS was not regulated whilst GOGAT was; however in *S. clavuligerus* (J71) and *S. noursei* (?) GOGAT was not regulated at all whilst GS was. Finally, in *S. coelicolor* A3(2) (A21) and *S. hygroscopicus* (A32) both enzymes were regulated by a nitrogen source.

3.6.3. Streptomycete Glutamate Dehydrogenases

GDH has been reported in a number of streptomycetes: *S. fradiae* (G68) (Romano and Nickerson, 1958; Vancurova *et al.*, 1989); *S. noursei* (?) (Grafe *et al.*, 1974b, 1977); *S. venezuelae* (A6) (Shapiro and Vining, 1983); *S. hygroscopicus* (A32) (Xia and Jiao, 1986); *S. cyanogenus* (?) (Watanabe *et al.*, 1976a); and *S. coelicolor* A3(2) (A21) (Fisher, 1989). However, no activity could be found in *S. clavuligerus* (J71) (Aharonowitz and Friedrich, 1980;

Brana *et al.*, 1986a) or *S. aureofaciens* (A31) (Vancurova *et al.*, 1988b). In *S. noursei* (?) the enzyme was $NADP^+$-dependent and activity was increased in cells where GS was low, and vice versa. Excess ammonium repressed GDH activity (Grafe *et al.*, 1979). High GDH activity correlated with the presence of ammonium in the culture medium for *S. venezuelae* (A6), *S. hygroscopicus* (A32) and *S. coelicolor* A3(2) (A21). The *S. venezuelae* (A6) and *S. coelicolor* A3(2) (A21) enzymes were NAD^+-dependent.

S. fradiae (G68) presented a special case. This streptomycete contained two GDH activities: one was $NADP^+$-dependent whilst the other was NAD^+-dependent. However, both isozymes were most active in cells grown on 25 mM ammonium. The purified $NADP^+$-dependent GDH had a tetrameric structure of 49 000 M_r. AMP, ADP and ATP were shown to inhibit both the forward and reverse reactions, with ATP having the most profound effect (Vancurova *et al.*, 1989). The NAD^+-dependent GDH was also purified and the deamination reaction found to be partially sensitive to adenyl nucleotides, but the reverse reaction was not (Nguyen *et al.*, 1997c).

3.6.4. Other Streptomycete Transaminases

As discussed in the introduction to this section, some attention has been focused on mechanisms of ammonium assimilation in streptomycetes using mechanisms other than GS–GOGAT and GDH–GOGAT. These studies have mainly centred on the role of alanine dehydrogenase (ADH). If this enzyme is to have a role, a transaminase – such as AOAT or perhaps alanine:2-isovalerate transaminase – needs to be present. Neither of these transaminases was found in *S. coelicolor* A3(2) (A21) (Fisher, 1989). No AOAT activity could be found in *S. clavuligerus* (Brana *et al.* 1986a). However, AOAT was reported to be present in *S. hygroscopicus* (A32), as was alanine hydrogenase (ADH) (Grafe *et al.*, 1974a). AOAT was also reported to be present in *S. noursei* (?) (Grafe *et al.*, 1974b), *S. cyanogenus* (?) (Watanabe *et al.*, 1976a) and *S. avermitilis* (?) (Novak *et al.*, 1992a).

Fisher (1989) reported that *S. coelicolor* A3(2) (A21) produced GOAT, which was unaffected by a nitrogen source. Thus ammonium could be assimilated via aspartate (Fig. 3), provided the strain generates aspartate. Aspartase (aspartate ammonium-lyase) catalyses the incorporation of ammonium into fumarate. However, no-one has reported the presence of this enzyme in *S. coelicolor* A3(2) (A21). Romano and Nickerson (1958) reported the presence of GOAT in *S. fradiae* (G68). However, they also reported that they could find neither aspartase nor aspartate dehydrogenase activities. These observations were taken to explain why aspartate could act as sole nitrogen source but not as sole carbon source for this streptomycete. GOAT was also reported to be present in *S. noursei* (?) by Grafe *et al.* (1974b).

3.6.5. Pathway(s) of Ammonium Assimilation in Different Streptomycetes

Several approaches have been taken to identify the pathways of nitrogen assimilation in streptomycetes. One approach has been to demonstrate that the appropriate enzymes are present and that they are regulated in the appropriate fashion. However, there is the danger of misinterpretation because the apparent co-regulation of enzymes may be an effect, not a means of ammonium assimilation. A case in point is *S. clavuligerus* (J71), where ADH was proposed as an enzyme of ammonium assimilation (Aharonowitz and Friedrich, 1980); but later work showed that as AOAT was missing (Brana *et al.*, 1986a) this could not be so. Another approach has been to isolate mutations in the genes encoding the appropriate enzymes and then to see whether the phenotype of the mutant is that to be expected if the enzyme has its proposed role. A more direct approach is the use of nitrogen isotope tracer studies to see which amino acids become labelled during nitrogen assimilation.

3.6.5.1. Streptomyces venezuelae *(A6).* ^{15}N nuclear magnetic spectroscopy was used to study ammonium assimilation in *S. venezuelae* (A6) (Shapiro *et al.*, 1985). ^{15}N ammonium was incorporated by exponential-phase cells mainly into glutamate–glutamine. In dense, stationary-phase cells and anoxic cells, ^{15}N alanine was the chief product. It was suggested that this was a way of storing nitrogen as alanine during poor growth. Another possibility was that ADH was being used to regenerate NAD^+ from NADH during substrate-level phosphorylation. ADH acted, therefore, as a substitute for lactate dehydrogenase, which the authors reported was not present in the strain. The implication is that, in growing cells, ammonium was assimilated via the GS–GOGAT couple and/or the GDH–GOGAT couple in this streptomycete.

3.6.5.2. Streptomyces clavuligerus *(J71).* The genetic approach has been used to study the assimilation pathways of this species (Brana *et al.*, 1986a; Bascaran *et al.*, 1989a, b). Two glutamine- and six glutamate-requiring mutants and an ADH mutant were isolated. The glutamate-requiring mutants could not grow on ammonium, or on amino acids presumed to be catabolized via ammonium, and they had no discernible GOGAT activity. The fact that these mutants could be isolated in a single step from wild type indicates that the only means of synthesizing glutamate using ammonium in *S. clavuligerus* (J71) is via GOGAT. This is consistent with the absence of GDH and AOAT. The observations that the two glutamine-requiring mutants had less than 5% of the wild-type activity of GS, and that growth of the mutants was not stimulated by addition of ammonium in the presence of glutamine, indicated that GS was the only way of assimilating ammonium. From the discussion above (see section 3.6.1) it would appear that these mutants were control mutants that inactivate both GSI and GSII.

3.6.5.3. Streptomyces coelicolor *A3(2) (A21).* Fisher (1989) isolated seven GOGAT mutants of this streptomycete. These mutants could not grow on asparagine, alanine, histidine or arginine as the sole nitrogen source, but they could grow on media containing ammonium, glutamate, glutamine, aspartate and proline as the sole nitrogen source. When one of these mutants was subjected to a further round of mutagenesis, three GDH mutants were isolated. These GOGAT GDH double mutants could not grow on media with ammonium as the sole nitrogen source, but grew on media with glutamine, glutamate, aspartate or proline as the sole source. These phenotypes were consistent with the proposal that this streptomycete use the GS–GOGAT couple under conditions where ammonium supply was low and the GDH–GOGAT couple where ammonium supply was plentiful.

3.6.5.4. S. avermitilis *(?).* As mentioned above (section 3.6.4), *S. avermitilis* contains an alanine aminotransferase (AOAT; Fig. 3), in addition to alanine dehydrogenase (ADH; Fig. 3) GS, GOGAT and GDH. This raises the possibility that, in addition to the GDH–GOGAT couple as a mechanism to assimilate ammonium at high ammonium levels, there may be an ADH–AOAT couple. At 7.5 mM ammonium, GS, GOGAT and GDH activities were high but ADH was low. At 75 mM ammonium, ADH activity increased nearly 1000-fold (Novak *et al.,* 1992a). It would be interesting to study the pathways of ammonium assimilation in *S. avermitilis* (?) using mutants in GDH and ADH and/or using ^{15}N tracer experiments.

3.7. Global Regulation of Nitrogen Metabolite Catabolism

3.7.1. Stringent Response

Many bacteria exhibit the stringent response. Starvation of the cell for amino acids (nutrient shift-down) leads to an increase in the intracellular concentration of the alarmones ppGpp and pppGpp. These nucleotides are responsible for the inactivation of transcription of the rRNA, tRNA and ribosomal protein operons, halt of cell wall growth and modification of various aspects of metabolism. The overall effect is to stimulate systems involved in supplying the new needs of the cell whilst inducing a transient cessation of growth. Therefore, the cell can adapt to grow at the maximum growth rate possible in the more limited resources available. Formation of the alarmones can also be induced by addition of amino acid analogues that inhibit charging of the appropriate tRNAs. It is believed that the presence of uncharged tRNAs interacting with the ribosome is the signal that activates synthesis of the alarmones by the ribosome-associated *relA* gene product (Cashel and Rudd, 1987).

Production of both ppGpp and pppGpp was identified in a number of

streptomycetes (An and Vining, 1978; Hamagishi *et al.*, 1981). During cultivation in complex liquid medium, exponential-phase *S. griseus* (A1B) cells contained relatively high levels of the alarmones, which decreased by five-fold as the cells entered the stationary phase. If early stationary-phase cells were transferred to glucose-, glycine- or phosphate-free medium, an immediate four-fold increase in ppGpp levels was observed, before decay back to basal levels. As the concentration of the nucleotides were high during exponential growth, the authors concluded that the medium was nutrient-limited even during the period of most rapid growth (An and Vining, 1978). A possible explanation of this effect was that some cells within microcolonies in the culture were starved whilst others were growing rapidly (section 1.7). The fragmentation-state of the culture was not reported. From the kinetics of ppGpp accumulation it was concluded that there was no correlation with initiation of secondary metabolite (streptomycin) synthesis.

S. hygroscopicus (A32) cells, which had just arisen from germinated spores and were undergoing balanced growth, accumulated ppGpp and pppGpp then dissipated the nucleotides following nutrient shift-down caused by addition of non-metabolizable serine hydroxamate (serine analogue) or methyl-α-D-glucopyranoside (glucose analogue) (Riesenberg *et al.*, 1984). Measurable amounts of neither nucleotide could be found during exponential growth. During serine hydroxamate treatment, the ATP and GTP pools decreased, the latter at a greater rate than the former. The CTP and UTP pools also decreased. Methyl-α-D-glucopyranoside treatment caused a transient decrease in the ATP pool, whilst the CTP pool fell and then recovered slightly. The GTP pool dropped and stayed low. The addition of either the glucose or serine analogue led to a rapid cessation of protein and RNA synthesis, and this was directly correlated with a cessation of cell growth. There was some evidence of limited recovery of the capacity for protein synthesis and cell growth in the case of carbon shift-down. Riesenberg *et al.* (1984) and An and Vining (1978) noted that the concentrations of ppGpp and pppGpp observed were greater than 100-fold less than those seen in enteric bacteria. This should be reflected in an increased sensitivity of the targets within streptomycetes, compared with enteric bacteria.

S. coelicolor A3(2) (A21) was shown to have a classic stringent response, which could be initiated either by nutrient shift-down or treatment with serine hydroxamate (Strauch *et al.*, 1991). In addition to ppGpp and pppGpp accumulation, ppGp was also accumulated with same kinetics as ppGpp. The GTP, ATP, UTP and CTP pools all decreased as the alarmone pools increased. At the end of exponential growth there was also a transient increase in ppGpp. The accumulation of the nucleotides was correlated with shutdown of the promoter of the *rrnD* rRNA operon, as measured by S1 nuclease mapping. The observations that nutritional shift-down induced transcription of actinorhodin genes, whilst serine hydroxamate addition did not and yet both induced ppGpp

accumulation, implied that the alarmone was not the crucial signal for initiation of secondary metabolism.

The same conclusion as for *S. coelicolor* A3(2) (A21) was reached following extensive study by Bascaran *et al.* (1991), who examined cephamycin synthesis in *S. clavuligerus* (J71). During further work on *S. clavuligerus* (J71), ppGpp concentration increased to low levels during carbon (glycerol) starvation but increased to high levels during phosphate starvation (Jones *et al.*, 1997). Previous work, under the conditions used, ppGpp was observed at low concentration in all cases, but guanosine 5′-diphosphate 3′-monophosphate, ppGp, concentration increased during nutrient shift-down (amino acid depletion). This triphosphate nucleotide was not synthesized from ppGpp and there was possible correlation with cephamycin production but not clavulanic acid production (Jones *et al.*, 1996).

The possible roles of ppGpp and pppGpp accumulation and concomitant GTP pool size decreases have been the subject of intensive study by Ochi using a number of different streptomycete strains (Ochi, 1988). In all cases examined, the strains had a classic stringent response. He suggested that ppGpp accumulation might be associated with initiation of secondary metabolism, whilst GTP concentration fall was associated with initiation of sporulation. Both of these views are controversial. (See Hodgson (1992) for a review of the role in sporulation.) Recent work with relaxed mutants, which have an impaired ability to synthesize ppGpp, has also raised objections to a simple interpretation (Ochi, 1990).

The cloning of genes whose products have roles in pppGpp and ppGpp synthesis from *S. coelicolor* A3(2) (A21) and *S. antibioticus* (A31) and the generation of mutants has in some aspects clarified, and in other aspects clouded, the interpretations from the physiological studies. The *S. coelicolor* A3(2) (A21) (p)ppGpp synthetase gene, *relA*, was isolated in two different ways: directed cloning using PCR (Chakraburtty *et al.*, 1996), and identification following sequencing of a DNA fragment that stimulated actinorhodin production when on a multicopy plasmid (Martinez-Costa *et al.*, 1996). The gene product had greater similarity to the *E. coli spoT* product, which is involved more in ppGpp degradation than synthesis. However, studies with a deletion mutant showed that the intact enzyme was ribosome-associated during (p)ppGpp synthesis *in vitro*. Moreover, when the C-terminus of the gene product was deleted, (p)ppGpp synthesis became ribosome-independent. Degradation of (p)ppGpp was associated with residues 93-397 while (p)ppGpp synthesis was associated with residues 267-453 (Martinez-Costa *et al.*, 1996, 1998).

The *relA* deletion mutant was slow-growing, delayed in sporulation and deficient in actinorhodin production, but unaffected in production of two other secondary metabolites. Addition of a wild-type copy of the streptomycete gene and expression of the *E. coli relA* gene compensated for all these

deficiencies. In addition, the streptomycete gene stimulated production of actinorhodin and undecylprodigiosin (Martinez-Costa *et al.*, 1996, 1998).

Disruption of the *relA* gene, while potentially retaining ribosome-independent (p)ppGpp synthesis and degradation capacity, again led to a slow-growth phenotype but there was no effect on sporulation or antibiotic synthesis (Chakraburtty *et al.*, 1996). When the *relA* gene was deleted, (p)ppGpp synthesis was abolished but there was no effect on growth rate but spore development was delayed and reduced. Secondary metabolism was conditionally affected. Production of both actinorhodin and undecylprodigiosin was abolished under conditions of nitrogen limitation but unaffected on other media (Chakraburtty and Bibb, 1997). A more detailed study of actinorhodin and undecylprodigiosin production under continuous culture conditions with different limitations indicated that the two secondary metabolites had been affected by the *relA* deletion in different ways. The general conclusion drawn was that there was 'an important and general role for the ppGpp in determining the onset of antibiotic biosynthesis under conditions of nutrient limitation.' (Kang *et al.*, 1998).

The situation in *S. antibioticus* (A31) was different from that in *S. coelicolor* A3(2) (A21). An enzyme responsible for ribosome-independent synthesis of pppGpp *in vitro* turned out, when its gene was cloned, to be a polynucleotide phosphorylase. The *in vitro* activity of the enzyme was stimulated by methanol and by addition of polyuridine plus transfer RNA. The *E. coli* polynucleotide phosphorylase was not capable of pppGpp synthesis, but the streptomycete enzyme was capable of pppGpp synthesis, and polymerization of ADP and phosphorolysis of polyadenosine (Jones, 1994a, b; Jones and Bibb, 1996). Nothing has been reported on the phenotype of a mutant in the streptomycete polynucleotide phosphorylase gene. It has also not been reported whether *S. coelicolor* A3(2) (A21) has a copy of the polynucleotide phosphorylase gene, or whether *S. antibioticus* (A31) has a copy of the streptomycete *relA* gene.

3.7.2. Catabolite Repression

3.7.2.1. Carbon Catabolite Repression. Catabolism of nitrogen-containing metabolites such as amino acids and bases might potentially be subject to a number of global control mechanisms. In enteric bacteria, carbon catabolite repression and nitrogen catabolite repression have been shown to control a large number of amino acid catabolism systems (Magasanik and Neidhardt, 1987). Carbon catabolite repression includes inducer exclusion and repression of gene expression by glucose and related monosaccharides. Glucose repression and inducer exclusion operate via the phospho*enol*pyruvate:PTS glucose transport system and repression is mediated by cyclic 3′,5′-AMP (section 2.4.3).

Carbon catabolite repression has been shown to be a global control

mechanism active in streptomycetes (section 2.4.5). The carbon catabolite with the most repressive effect is often, but not exclusively, glucose. A number of proteases and amino acid catabolism systems have been shown to be subject to carbon catabolite repression (Table 12). However, proline and histidine catabolism were not subject to repression in a streptomycete known to have a glucose repression system; i.e. *S. coelicolor* A3(2) (A21). In enteric bacteria, proline and histidine catabolism are paradigmatic of glucose repression of amino acid catabolic systems. It is now clear that the glucose repression of valine dehydrogenase induction noted in *S. coelicolor* A3(2) (A21) is due to the same mechanism as glucose repression of carbohydrate metabolism, i.e. it involves the *glkA* gene (Tang and Hutchinson, 1995). In conclusion, it appears that carbon catabolite repression does not play as large a role in control of amino acid metabolism in streptomycetes as it does in other bacteria.

3.7.2.2. Nitrogen Catabolite Repression. As stated in the introduction to section 3 a great deal of interest has been shown in the mechanisms of nitrogen catabolite repression in streptomycetes because of the practical problem of 'nitrogen repression' of secondary metabolism. It has been demonstrated that nitrogen repression really is important in control of primary metabolism, as a number of proteases, nitrogen assimilation systems and amino acid catabolism systems were repressed. Ammonium was often the best repressing agent (Table 13). I shall describe briefly the nitrogen catabolite repression system in enteric bacteria, because this is the system best characterized at the molecular level, and then discuss what we know in streptomycetes.

Nitrogen catabolite repression in enteric bacteria involves the *ntr* regulon. The members of the regulon include amino acid catabolic systems and are characterized by a common requirement for a phosphorylated activator protein, NtrC, and σ^{54} RNA polymerase. Sigma factor σ^{54}, the product of *rpoN*, allows the RNA polymerase to recognize a specific promoter consensus sequence. This form of RNA polymerase is required not just for the *ntr* regulon, but also for many other positively activated genes. As well as these global requirements, the cognate activator molecule, usually the amino acid catabolite or a derivative, activated each member of the *ntr* regulon. The balance of α-ketoglutarate to glutamine within the cell was perceived by the product of the *glnD* gene, GlnD. A low glutamine:high α-ketoglutarate ratio stimulated GlnD to uridylylate (attach UMP to) P_{II}, the product of the *glnB* gene. P_{II}-UMP ceases interaction with NtrB (*ntrB* product), which then acts as a protein kinase and phosphorylates the *ntrC* product, NtrC. Phosphorylated NtrC is an activator of gene transcription in the *ntr* regulon. A high glutamine:low α-ketoglutarate ratio stimulates GlnD to remove the uridylyl group from P_{II}-UTP. P_{II} associates with NtrB, which converts the protein kinase action of NtrB to a phosphoprotein phosphatase; dephosphorylation of phosphorylated NtrC results, and thus down-regulation of the *ntr* regulon (Magasanik and Neidhardt, 1987).

Table 12 Carbon catabolite repression of nitrogen metabolite catabolism enzymes in streptomycetes.

System	Strain	Repressor	Fold repression	Reference
Protease	*S. aureofaciens* (A14)	Glucose	67–134	Laluce and Molinari, 1977
	S. lividans (A21)	Glucose	*c.* 3	Aretz *et al.*, 1989
	S. spheroides (A1B)	Glucose	2.5	Al-Nuri *et al.*, 1986
	Streptomyces sp. C5	None		Gibb *et al.*, 1989
Nitrate reduction	*S. fulvoviridis* (A3)	Sorbose	100%	Mansour and Shady, 1984
	Sucrose	100%		
Cadaverine aminotransferase	*S. clavuligerus* (J71)	Glycerol	2	Madduri *et al.*, 1991
Histidine catabolism	*S. coelicolor* A3(2) (A21)	None		Kendrick and Wheelis, 1982
	S. griseus (A15)	None		Kroening and Kendrick, 1987
Proline catabolism	*S. coelicolor* A3(2) (A21)	None		Smith *et al.*, 1995
Valine dehydrogenase	*S. ambofaciens* (A23)	Glycerol	?	Lounes *et al.*, 1995b
	S. coelicolor (A21)	Glucose	3.5	Navarrete *et al.*, 1990

Excluded are 'catabolic enzymes' that are believed to be involved in secondary metabolism rather than amino acid catabolism.

Table 13 Nitrogen catabolite repression of nitrogen metabolite catabolism enzymes in streptomycetes.

System	Strain	Repressor	Fold repression	Reference
Protease	*S. aureofaciens* (A14)	Ammonium	100%	Laluce and Molinari, 1977
	S. clavuligerus (J71)	Ammonium	5-6.5	Bascaran *et al.*, 1990b
	S. spheroides (A1B)	Ammonium	2.22	Al-Nuri *et al.*, 1986
		Nitrate	2	
	Streptomyces sp. SMF	Ammonium	100%	Shin and Lee, 1986
		Cysteine	3.5	
Nitrate transport	*S. venezuelae* (A6)	Ammonium	?	Shapiro and Vining, 1984
Nitrate reduction	*S. fulvoviridis* (A3)	Ammonium	2	Mansour and Shady, 1984
	S. venezuelae (A6)	None		Shapiro and Vining, 1984
Urease	*S. clavuligerus* (J71)	Ammonium	>23	Bascaran *et al.*, 1989a
Glutamine synthetase	*S. cattleya* (C47)	Ammonium	8	Paress and Streicher, 1985
	S. clavuligerus (J71)	Ammonium	6.4	Bascaran *et al.*, 1989a
	S. coelicolor A3(2) (A21)	Amino acids	6	Fisher and Wray, 1989
		+ ammonium	56.8	Nguyen *et al.*, 1994
	S. hygroscopicus (A32)	Ammonium	6	Xia and Jiao, 1986
	S. venezuelae (A6)	None		Shapiro and Vining, 1983
Glutamate synthase	*S. clavuligerus* (J71)	None		Brana *et al.*, 1986a
	S. coelicolor A3(2) (A21)	Amino acids	7	Fisher, 1989
	S. venezuelae (A6)	Proline	27	Shapiro and Vining, 1983
		Isoleucine	20	

Table 13 *cont.*

System	Strain	Repressor	Fold repression	Reference
Glutamate dehydrogenase	*S. coelicolor* A3(2) (A21)	Amino acids	10	Fisher, 1989
	S. fradiae (G68)	Alanine	4.9	Vancurova *et al.*, 1989
	S. noursei (?)	Ammonium	6.3	Grafe *et al.*, 1979
	S. venezuelae (A6)	Isoleucine	100%	Shapiro and Vining, 1983
Arginase	*S. clavuligerus* (J71)	Ammonium + glutamine	3.3	Bascaran *et al.*, 1989a
Ornithine aminotransferase	*S. clavuligerus* (J71)	Ammonium + glutamine	3.2	Bascaran *et al.*, 1989a
Cadaverine aminotransferase	*S. clavuligerus* (J71)	Ammonium	1.5	Madduri *et al.*, 1991
		Glutamate	1.6	
Histidine catabolism	*S. clavuligerus* (J71)	None		Bascaran *et al.*, 1989a
	S. coelicolor A3(2) (A21)	None		Kendrick and Wheelis, 1982
	S. griseus (A15)	None		Kroening and Kendrick, 1987
Proline transport	*S. clavuligerus* (J71)	None		Bascaran *et al.*, 1990a
	S. coelicolor A3(2) (A21)	None		Hood *et al.*, 1992
	S. venezuelae (A6)	Ammonium	?	Shapiro and Vining, 1984
		Nitrate	?	
Proline catabolism	*S. clavuligerus* (J71)	None		Bascaran *et al.*, 1989a
	S. coelicolor A3(2) (A21)	None		Hood *et al.*, 1992
	S. niveus (A1B)	Ammonium	100%	Kominek, 1972
Tyrosinase	*S. michiganensis* (A6)	Ammonium	100%	Held and Kutzner, 1990
Valine dehydrogenase	*S. aureofaciens* (A14)	None		Nguyen *et al.*, 1995a
	S. fradiae (G68)	Ammonium	5.8	Omura *et al.*, 1983b
			5.1	Vancura *et al.*, 1987

Excluded are 'catabolic enzymes' that are believed to be involved in secondary metabolism rather than amino acid catabolism.

A systematic analysis of nitrogen catabolite regulation of nitrogen catabolism enzymes was made in *S. clavuligerus* (J71) (Bascaran *et al.*, 1989a, 1990b). Enzymes involved in serine, proline and histidine catabolism were not subject to ammonium repression, whilst the latter two were induced by the cognate catabolite. Glutamine synthetase (GS), urease, a 41.7 kDa protease and arginine catabolic enzymes were subject to ammonium repression. Mutants were isolated which were deregulated for this ammonium repression. They were selected on the basis that derepression had occurred for urease and GS and then screened for the effect on two putative arginine catabolic enzymes (OAT and arginase) and the protease. None of the mutants were auxotrophs and they affected the five enzymes differentially (Fig. 4) (Bascaran *et al.*, 1989b, 1990b). One mutant, M31, was derepressed for urease, protease, OAT and arginase but contained less GS activity, which was still subject to very mild

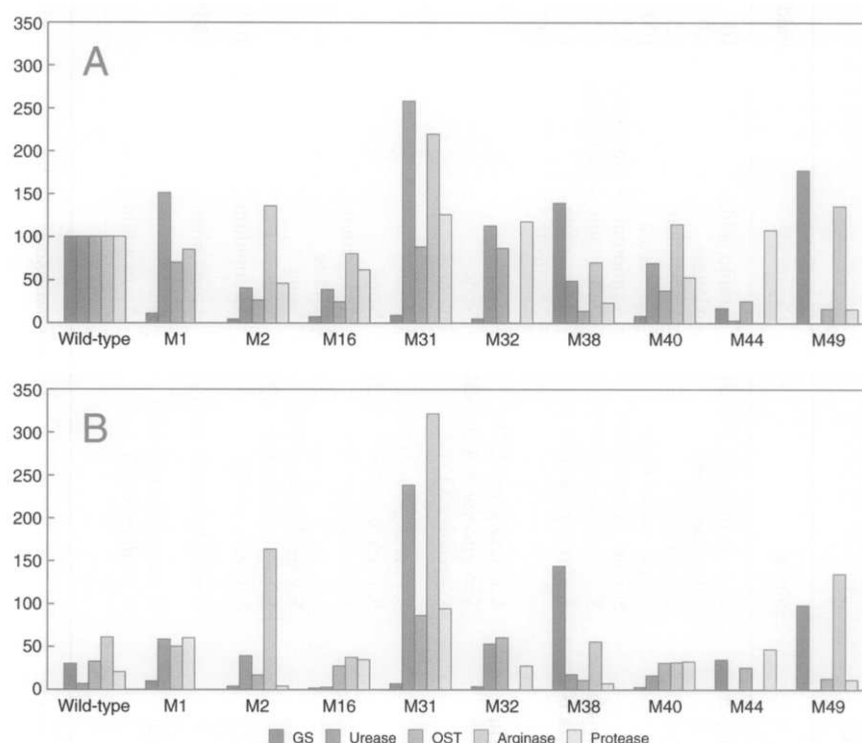


Figure 4 Nitrogen catabolism regulation of wild type and mutants of *Streptomyces clavuligerus* (J71): (A) in absence of ammonium; (B) in presence of ammonium. The vertical axis shows the relative enzyme activity (activity of wild type in absence of ammonium taken as 100%). GS = glutamine synthetase; OAT = ornithine aminotransferase. (Data from Bascaran *et al.*, 1989b, 1990b).

suppression by ammonium. Another mutant, M49, contained increased GS activity, which was still subject to ammonium repression, and increased ammonium-resistant arginase but decreased urease, OAT and protease activities. It appeared that three of the enzymes were fully repressed in the absence of ammonium. It was noted that the GS activity of seven of the nine mutants had become thermolabile. As discussed in section 3.6.1, this was a characteristic of GSII and it might imply that the seven mutants had lost GSI activity but retained GSII activity. Total GS activity was decreased in six of the seven mutants.

The genes affected in the nine *S. clavuligerus* (J71) mutants have not been further characterized. Most importantly, all of these mutants that had become deregulated for nitrogen catabolite repression of primary metabolism still retained 'nitrogen repression' of secondary metabolite, cephalosporin, biosynthesis.

Mutants affected in nitrogen regulation of primary metabolism have been isolated in *S. coelicolor* A3(2) (A21). Mutants that had lost GOGAT (*glt*) were essentially unaffected in glutamate dehydrogenase (GDH), glutamate:oxaloacetate transaminase (GOAT) and alanine dehydrogenase (ADH). Double mutants that had lost GOGAT and GDH (*glt5 gdh5*) produced slightly more GOAT (1.5-fold) and 35-fold more ADH. It was proposed that this increased ADH activity was due to the accumulation of ammonium in the $GOGAT^-$ GDH^- cells, although addition of ammonium to wild-type cells did not stimulate ADH activity. A surprising observation was that two of the *glt* mutants were unable to induce histidinase activity, and genetic crosses could not separate the GOGAT phenotype from the Hut phenotype. This might imply that this class of *glt* mutant contained global regulatory mutations, even though they mapped to the same segment of the chromosome as the other *glt* mutations (Fisher, 1989).

A number of mutations (*gln*) that affect expression of GS activity have been isolated in *S. coelicolor* A3(2) (A21) (Wray *et al.*, 1991) and *S. clavuligerus* (J71) (Brana *et al.*, 1986a). As there was evidence of two GS activities in streptomycetes, GSI and GSII, it must be assumed that these mutations affect a gene(s) that is required for expression of both types (section 3.6.1). In the case of *S. coelicolor* A3(2) (A21), all six *gln* mutants mapped to a single gene that has been cloned and sequenced. The gene, *glnR*, had no homology to *glnA* (GSI structural gene) or *glnII* (GSII structural gene). Its product showed sequence similarity to OmpR and PhoB, which are gene activator components of two component systems in *E. coli*, concerned with osmotic pressure regulation and phosphate metabolism, respectively (Wray and Fisher, 1993). They belong in the same family as NtrC, which is involved in nitrogen catabolite regulation (see above). It is not clear whether *glnR* was required for expression of any other nitrogen metabolism genes in *S. coelicolor* A3(2) (A21).

Whilst studying genetic instability and DNA amplification in *S. lividans* (A21), a number of strains were identified that had lost the ability to grow on asparagine as sole nitrogen source but could use glutamate or ammonium. Dyson and Schrempf (1987) classified this as a 'defect in nitrogen assimilation (Ntr)', which implied a pleiotropic effect; however, no evidence was presented to justify this. A simpler interpretation is that the strains carried a lesion in asparagine catabolism, such as loss of asparaginase.

Attempts were made to find a global nitrogen catabolite regulation system in *S. venezuelae* (A6) but no evidence for this or repression of chloramphenicol production could be found (Shapiro and Vining, 1985).

3.8. Nitrogen Metabolite Catabolism: General Conclusions

Evidence has been presented of amino acid catabolism systems that are induced by the cognate catabolite or a metabolite (Table 14, Fig. 5). The levels of induction are in the same range as those for carbohydrate catabolism (Table 10, Fig. 1). However, in some cases amino acid catabolism enzymes were expressed constitutively (Table 14, Fig. 5).

Interestingly, enzymes that were used in amino acid catabolism in other bacteria were exclusively involved in synthesis of secondary catabolites. Examples include: (1) tryptophan dioxygenase, kynurenine formamidase II and hydroxykynureninase for actinomycins in *S. parvulus* (A12) (section 3.3.1.1); (2) histidine aminotransferase for nikkomycins in *S. tendae* (A12) (section 3.3.2); (3) arginine amidinotransferase for streptomycin in *S. griseus* (A15) (section 3.3.3.2); (4) arginase and ornithine aminotransferase for clavulanic acid in *S. clavuligerus* (J71) (section 3.3.3.2); and (5) lysine ε-aminotransferase for β-lactams in *S. clavuligerus* (J71) (section 3.3.5.4). In several of these streptomycetes there was a different pathway for amino acid catabolism that was regulated by induction with the cognate amino acid. In the lysine case, the gene encoding the amino acid-modifying enzyme involved in secondary catabolism has been shown to be clustered with the secondary metabolite biosynthesis genes and to be co-regulated with them. This serves as an object lesson in how careful we must be before we assume an enzyme is in either a primary or secondary metabolic pathway.

Nitrogen catabolite regulation of secondary metabolism is an important consideration when attempting to produce secondary metabolites on a commercial scale. In one case, valine dehydrogenase (VDH) and tylosin production (section 3.3.4), the evidence is very strong that the reason for 'nitrogen repression' of antibiotic biosynthesis was ammonium suppression of activity of VDH. This primary metabolic enzyme was responsible for precursor supply. It was not clear whether the ammonium inhibited enzyme action by product feedback inhibition or whether it repressed enzyme expression.

Table 14 Levels of induction of amino acid catabolic systems in streptomycetes.

System	Inducer	Induction level	Strain	Reference
Alanine dehydrogenase	Alanine	37.6	*S. clavuligerus* (J71)	Aharonowitz and Friedrich, 1980
	Alanine	84.7	*S. clavuligerus* (J71)	Brana *et al.*, 1986a; 1986b
	Alanine	106.9	*S. coelicolor* A3(2) (A21)	Fisher, 1989
	Alanine	78.5	*S. phaeochromogenes* (A40)	Itoh and Morikawa, 1983
	Alanine	86	*S. avermitilis* (?)	Novak *et al.*, 1997
	Ammonium	5	*S. avermitilis* (?)	Novak *et al.*, 1997
Alanine:2-oxoglutarate transaminase	Alanine	3-4	*S. avermitilis* (?)	Novak *et al.*, 1997
	Ammonium	3-4	*S. avermitilis* (?)	Novak *et al.*, 1997
Arginase	Arginine	6.7	*S. clavuligerus* (J71)	Bascaran *et al.*, 1989a
	Arginine	2	*S. clavuligerus* (J71)	Padilla *et al.*, 1991
	Arginine	1.2	*S. coelicolor* A3(2) (A21)	Padilla *et al.*, 1991
	Arginine	2.3	*S. griseus* (A15)	Padilla *et al.*, 1991
	Arginine	2.6	*S. lividans* (A21)	Padilla *et al.*, 1991
Arginine oxidase	Arginine	>26.5	*S. griseus* (A15)	Thoai *et al.*, 1966
Ornithine aminotransferase	Arginine	4.4	*S. clavuligerus* (J71)	Bascaran *et al.*, 1989a
γ-guanidinobutyramide amidase	Arginine	>67.4	*S. griseus* (A15)	Thoai *et al.*, 1966
γ-guanidinobutyrate ureohydrolase	Arginine	32	*S. clavuligerus* (J71)	Padilla *et al.*, 1991
	Arginine	1.8	*S. coelicolor* A3(2) (A21)	Padilla *et al.*, 1991
	Arginine	16.3	*S. griseus* (A15)	Padilla *et al.*, 1991
	Arginine	20	*S. lividans* (A21)	Padilla *et al.*, 1991

Table 14 cont.

System	Inducer	Induction level	Strain	Reference
Asparaginase	Asparagine	5.8	*S. karnatakensis* (C44)	Mostafa *et al.*, 1979
	Asparagine	6.7	*S. venezuelae* (A6)	
Histidase	Histidine	41	*S. clavuligerus* (J71)	Bascaran *et al.*, 1989a
	Histidine	None	*S. coelicolor* A3(2) (A21)	Wu *et al.*, 1992a
	Histidine	None	*S. griseus* (A15)	Wu *et al.*, 1992a
Urocanase	Urocanate	3.8	*S. clavuligerus* (J71)	Bascaran *et al.*, 1989a
	Urocanate	>190	*S. coelicolor* A3(2) (A21)	Kendrick and Wheelis, 1982
	Urocanate	>67	*S. griseus* (A15)	Kroening and Kendrick,1989
Imidazolonepropionate hydrolase	Urocanate	>12.4	*S. coelicolor* A3(2) (A21)	Kendrick and Wheelis, 1982
Formiminoglutamate iminohydrolase	Urocanate	>9.2	*S. coelicolor* A3(2) (A21)	Kendrick and Wheelis, 1982
	Urocanate	>63	*S. griseus* (A15)	Kroening and Kendrick,1989
Cadaverine aminotransferase	Lysine	3–7.3	*S. clavuligerus* (J71)	Madduri *et al.*, 1991
	cadaverine	4.5		
Methionine decarboxylase	Methionine	2.6	*Streptomyces* sp. K37	Hagino and Nakayama, 1968
Homogentisate 1,2-dioxygenase	PhenylAlanine	3.1	*S. badius* (C single)	Pometto and Crawford, 1985
	Tyrosine	6.2		
	PhenylAlanine	11.7	*S. setonii* (A1B)	
	Tyrosine	11.2		
	Phenylalanine	3.2	*S. sioyaensis* (A29)	
	Tyrosine	4.9		
	Phenylalanine	>10	*S. viridosporus* (A15)	
	Tyrosine	>3		
	Phenylalanine	1.2	*Streptomyces* sp. V7	
	Tyrosine	13		

Table 14 cont.

System	Inducer	Induction level	Strain	Reference
Proline oxidase	Proline	11.7	*S. clavuligerus* (J71)	Bascaran *et al.*, 1989a
Δ-Pyrroline-5-carboxylate dehydrogenase	Proline	300	*S. coelicolor* A3(2) (A21)	Smith *et al.*, 1995
Threonine dehydratase	Threonine	2.1	*S. fradiae* (G68)	Vancura *et al.*, 1988b
Threonine aldolase	threonine	2.1	*S. fradiae* (G68)	Vancura *et al.*, 1988b
Tryptophan dioxygenase	Tryptophan	None	*S. parvulus* (A12)	Hitchcock and Katz, 1988
Kynurenine formamidase	Tryptophan	None	*S. parvulus* (A12)	Brown *et al.*, 1986
Kynureninase	Tryptophan	35	*S. parvulus* (A12)	Troost *et al.*, 1980
Hydroxykynureninase	Tryptophan	18	*S. parvulus* (A12)	Troost *et al.*, 1980
Valine dehydrogenase	Valine	5.2	*S. ambofaciens* (A23)	Lounes *et al.*, 1995a
I	Valine	16.3	*S. aureofaciens* (A14)	Vancurova *et al.*, 1988c
II	Valine	49.5	*S. aureofaciens* (A14)	Nguyen *et al.*, 1995b
	Valine	5.4	*S. avermitilis* (?)	Nguyen *et al.*, 1995d
	Valine	>100	*S. cinnamonensis* (?)	Priestly and Robinson, 1989
	Valine	13	*S. coelicolor* A3(2) (A21)	Navarrete *et al.*, 1990
	Valine	8.6	*S. fradiae* (G68)	Nguyen *et al.*, 1995a
α-ketoisovalerate dehydrogenase	Valine	6	*S. ambofaciens* (A23)	Lounes *et al.*, 1995a

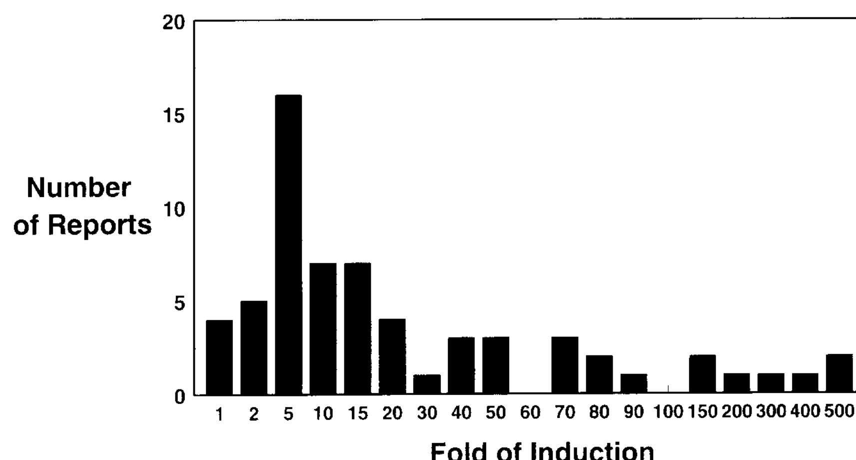


Figure 5 Induction of amino acid catabolism in streptomycetes (see Table 14 for data and references).

What was clear was that very high levels of ammonium were required to repress secondary metabolism. If the effect was via enzyme inhibition, we cannot hold out much hope for the isolation of ammonium-resistant mutants, because product feedback inhibition is an innate feature of the enzyme. The other implication from this work was that if the precursors were not present, the secondary metabolite genes were not activated. This further implied that precursor supply was a major means of activation of secondary metabolism. The same implication was noted from our work on proline and undecylprodigiosin production (Hood *et al.*, 1992) (section 3.3.3.1). However, one should not therefore conclude that addition of precursor to a culture will induce greater secondary metabolite production. The precursor may not be imported into the cell and, if it is, it may induce catabolism systems rather than be channelled into secondary metabolism. A better approach is to disrupt precursor catabolism.

Nitrogen catabolite regulation of β-lactam synthesis is a well-established phenomenon (Brana and Demain, 1988). However, the site(s) of action affected by the nitrogen catabolite(s) is unclear. It was demonstrated that the presence of ammonium in the culture medium 'repressed' the appearance of a number of enzymes of cephem synthesis in *S. clavuligerus* (J71) (Zhang *et al.*, 1989). However, it was not clear that this was a direct effect, or due to the inhibition of precursor supply, as in tylosin production. A mutant defective in LAT was unable to induce the genes encoding ACV synthetase and isopenicillin N synthetase, and this enzyme was subject to ammonium repression (Yu *et al.*, 1994). LAT was not involved in lysine catabolism in this strain. Loss of

nitrogen catabolite repression of primary metabolism had no effect on 'ammonium repression' of secondary metabolism in this strain (Bascaran *et al.*, 1989b).

The overall conclusion concerning amino acid catabolism in streptomycetes is that we have a lot of examples of regulation and non-regulation. It is clear that streptomycetes regulate their pathways in a different fashion from other bacteria. As we accumulate more information it is my belief that we will be able to exploit that information in terms of streptomycete productivity. At the present time we are just beginning to see how unusual is the control of streptomycete physiology.

4. NITROGEN METABOLITE BIOSYNTHESIS

4.1. Amino Acid Anabolism and its Control

4.1.1. The Aromatic Amino Acid Family

The common aromatic amino acid or shikimate pathway operates in all streptomycetes tested. This pathway results in the formation of chorismate that is the common precursor of the tryptophan, phenylalanine and tyrosine pathways and *p*-aminobenzoic acid, the precursor of tetrahydrofolic acid synthesis. It is also the precursor of NAD^+, which is derived directly from tryptophan, at least in *S. antibioticus* (A31) (Lingens and Vollprecht, 1964) (Fig. 6). A large literature exists concerning the aromatic amino acid pathways of streptomycetes because a number of important secondary metabolites are derived from intermediates and products of these pathways; e.g. chloramphenicol, actinomycin D and candicidin.

4.1.1.1. The Shikimate Pathway. The enteric bacteria contain three isozymes of the first enzyme of the common pathway, DAHP (3-deoxy-D-arabinoheptulosonate-7-phosphate) synthase. A different affector regulates each of these isozymes. In streptomycetes the presence of only one form of this enzyme has been reported. A number of laboratories have examined the enzyme and its regulation and these reports are summarized in Table 15. In general it appears that the enzyme is regulated poorly, if at all, at the gene expression level, the exception being *S. antibioticus* (A31) (Murphy and Katz, 1980).

Comparison of peptide sequences from the purified DAHP synthases of *S. rimosus* (B42) and *S. coelicolor* A3(2) (A21) were used to identify a previously sequenced *S. lividans* (A21) partial gene (L13767) as one for DAHP synthase; i.e. *aroH*. This *aroH* was found not to be homologous to other bacterial DAHP

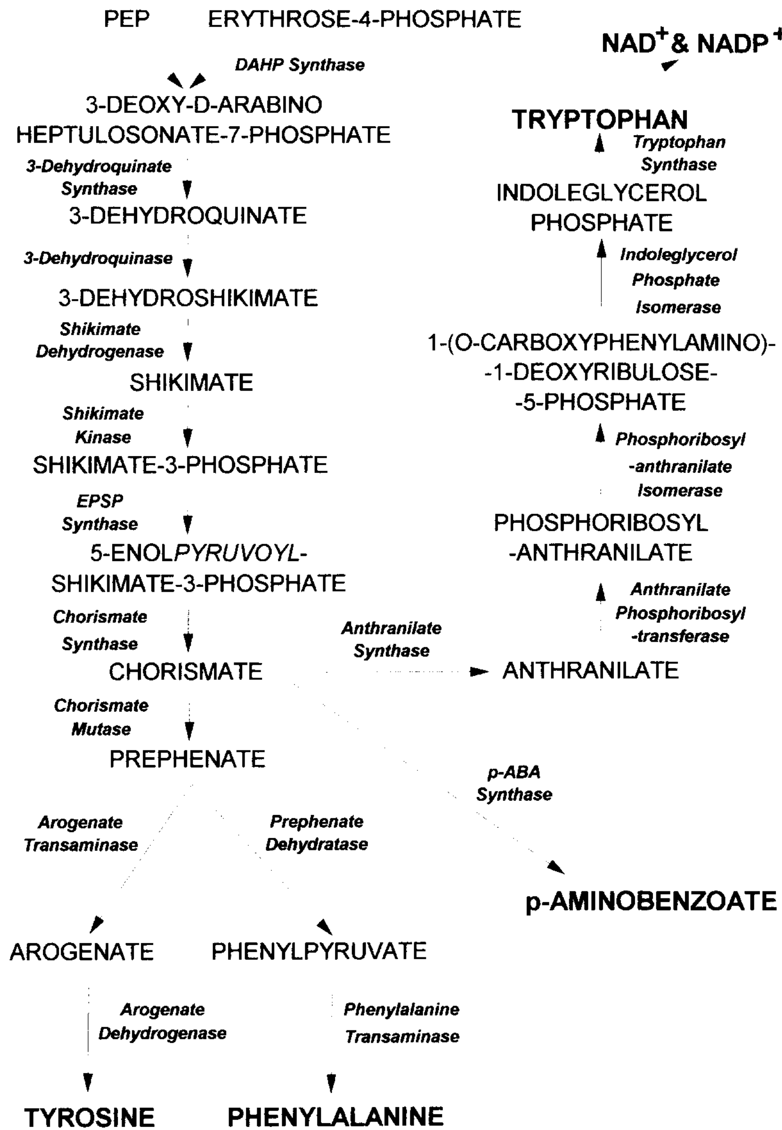


Figure 6 Pathways of aromatic amino acid biosynthesis in streptomycetes (see text for references).

Table 15 Regulation of aromatic amino acid biosynthesis in streptomycetes.

Enzyme	Enzyme regulation		Gene regulation		Strain	Reference
	Inhibitor	Inhibition	Inducer	Fold-repression		
DAHP synthase	Tryptophan	39%	?		*S. acidomyceticus* (?)	
	Tryptophan	58%	?		*S. albidoflavus* (A1A)	
	Tryptophan	23%	?		*S. endus* (A32)	Jensen *et al.*, 1967[a]
	Tryptophan	49%	?		*S. griseus* (A15)	
	Tryptophan	2%	?		*S. virginiae* (F61)	
	Tryptophan	65%	?		*S. aureofaciens* (A14)	Gorisch and Lingens, 1971
			p-Hydroxybenzoate	3		
	?		anthranilate	2	*S. venezuelae* (A6)	Lowe and Westlake, 1971
			p-Aminobenzoate	1.5		
			aromatic amino acids	1		
	Tryptophan	57%				
	Chorismate	1%	Tryptophan	11	*S. antibioticus* (A31)	Murphy and Katz, 1980
	Tryptophan + chorismate	80%				
	Tryptophan	80%	?		*S. rimosus* (B42)	Stuart and Hunter, 1993
	Tryptophan	67%	?		*S. coelicolor* A3(2) (A21)	Walker *et al.*, 1996
Chorismate mutase	None	None	?		*S. venezuelae* (A6)	Lowe and Westlake, 1972
			?			Gorisch and Lingens, 1972
	None	None			*S. aureofaciens* (A14)	Gorisch and Lingens, 1973

Table 15 Cont.

Enzyme	Enzyme regulation		Gene regulation		Strain	Reference
	Inhibitor	Inhibition	Inducer	Fold-repression		
Arogenate dehydrogenase	Tyrosine	73%	?		*S. antibioticus* (A31)	Keller *et al.*, 1983
	Tyrosine	77%	?		*S. arenae* (A18)	
	Tyrosine	83%	?		*S. glaucescens* (A28)	
	Tyrosine	77%	?		*S. gradiae* (?)	
	Tyrosine	55%	?		*S. griseus* (A15)	
	Tyrosine	0%	?		*S. lavendulae* (F61)	
	Tyrosine	55%	?		*S. niveoruber* (?)	
	Tyrosine	67%	?		*S. olivaceus* (A1C)	
	Tyrosine	72%	?		*S. parvulus* (A12)	
	Tyrosine	75%	?		*S. viridochromogenes* (A27	
Prephrenate dehydratase	Phenylalanine	100%	None		*S. venezuelae* (A6)	Lowe and Westlake, 1972
	Phenylalanine	90–98%	?		*S. refuineus* (?)	Speedie and Park, 1980
	Phenylalanine	0%	?		*S. antibioticus* (A31)	
	Phenylalanine	18%	?		*S. arenae* (A18)	
	Phenylalanine	28%	?		*S. glaucescens* (A28)	
	Phenylalanine	42%	?		*S. gradiae* (?)	
	Phenylalanine	41%	?		*S. griseus* (A15)	Keller *et al.*, 1983
	Phenylalanine	40%	?		*S. lavendulae* (F61)	
	Phenylalanine	0%	?		*S. niveoruber* (?)	
	Phenylalanine	60%	?		*S. olivaceus* (A1C)	
	Phenylalanine	49%	?		*S. parvulus* (A12)	
	Phenylalanine	0%	?		*S. viridochromogenes* (A27	

Table 15 *Cont.*

Enzyme	Enzyme regulation		Gene regulation		Strain	Reference
	Inhibitor	Inhibition	Inducer	Fold-repression		
Anthranilate synthase	Tryptophan	50%[b]	Tryptophan	5	*S. parvulus* (A12)	Katz *et al.*, 1984
			Tryptophan	12[f]		
	Tryptophan	50%[c]	Tryptophan	1.8–2.3		
	Anthranilate	50%[d]	Histidine	1.5–1.8	*S. venezuelae* (A6)	Lowe and Westlake, 1972
			p-aminobenzoate	1.5–1.7		Francis *et al.*, 1978
			Anthranilate	1.4–1.5		
	Tryptophan	44%[e]	Tryptophan	9.5[g]		Lin *et al.*, 1998
Anthranilate phosphoribosyl transferase	?		None		*S. coelicolor* A3(2) (A21)	Hu *et al.*, 1999
Indoleglycerol phosphate synthetase	?		None		*S. coelicolor* A3(2) (A21)	Hu *et al.*, 1999
Tryptophan synthase	?		None		*S. coelicolor* A3(2) (A21)	Hu *et al.*, 1999

[a]Jenson and Rebello (1970) reported that these observations were an artefact of the assay; [b]30 µmol of tryptophan. [c]10 nmol of tryptophan; [d]122 nmol of anthranilate; [e]42 mM tryptophan; [f]repression in a *trpD* bradytroph; [g]repression in a *trpC* mutant.

synthase genes but rather to be of a family of DAHP synthase genes previously found only in plants (Walker *et al.*, 1996). The homologue of the partial *S. lividans aroH* has been completely sequenced in *S. coelicolor* A3(2) (AL109661) and has been identified as encoding a plant-like DAHP synthase. Surprisingly, when the N-terminus of this gene was compared with the peptide fragment from the purified tryptophan-sensitive DAHP synthase purified from *S. coelicolor* A3(2) by Walker *et al.* (1996), there was complete agreement for the 13 most terminal residues but six of the remaining eight were completely different. This implies that there is another *aroH* gene present in *S. coelicolor* A3(2) and it may produce a DAHP synthase subject to different regulation. A second *aroH* gene of the plant-type has been found but it is clearly not the Walker *et al.* (1996) gene and is associated with a secondary *trp* cluster that might be involved in secondary metabolism (see section 4.1.1.4).

Tryptophan is the most common regulator of enzyme activity. There was evidence of cooperative inhibition of DAHP synthase by tryptophan and chorismate in *S. antibioticus* (A31) (Table 15). Jensen and Rebello (1970) reassessed the work of Jensen *et al.* (1967) and concluded that there was no regulation of DAHP synthase by tryptophan in the six streptomycetes retested, and that what had been observed previously was inhibition of the enzyme assay system by tryptophan. Other workers have confirmed the presence of tryptophan inhibition in the other streptomycete enzymes listed in Table 15.

White *et al.* (1990) reported that, in *S. coelicolor* A3(2) (A21), the third enzyme of the shikimate pathway, 3-dehydroquinate dehydratase or 3-dehydroquinase, had the characteristics of the catabolic, quinate-inducible, type-II, 3-dehydroquinase of fungi rather than the anabolic, constitutive 3-dehydroquinases of either fungi or bacteria. Evidence could not be found of other enzymes of a quinate degradation pathway in this streptomycete, nor could the bacterium grow on quinate. This co-option of a 'catabolic' type-II enzyme has been found in another streptomycete *S. hygroscopicus* var. *ascomyceticus* (A32) (Florova *et al.*, 1998) and a couple of actinomycetes, *Amycolatopsis methanolica* (Euverink *et al.*, 1992) and *Mycobacterium tuberculosis* (Garbe *et al.*, 1991).

4.1.1.2. Phenylalanine. Once chorismate has been formed it serves as a precursor of: prephenate via chorismate mutase; anthranilate via anthranilate synthetase; and *p*-aminobenzoate via *p*-aminobenzoate synthase. Chorismate mutase, the first enzyme of the branched phenylalanine and tyrosine pathways, has been examined in two streptomycetes (Table 15) and in each case no regulation at either the gene or enzyme level was observed.

The first dedicated enzyme of phenylalanine synthesis is prephenate dehydratase. In *S. venezuelae* (A6), the enzyme was not repressed by any of a wide range of products or intermediates of the aromatic amino acid pathways tested (Table 15). However, phenylalanine effectively inhibited enzyme activity at

physiological concentrations (Lowe and Westlake, 1972) and *S. refuineus* (?) (Speedie and Park, 1980). Keller *et al.* (1983) looked at a wide range of streptomycetes (Table 15), and identified three main classes of streptomycete. *S. antibioticus* (A31) and *S. viridochromogenes* (A27) possessed enzymes that were insensitive to both phenylalanine and tyrosine. The *S. niveoruber* (?) enzyme was insensitive to phenylalanine but was *stimulated* by tyrosine to 148% of the control activity. All the other streptomycetes tested (Table 15) contained enzymes that were insensitive to tyrosine but were inhibited to greater or lesser extent by phenylalanine. None of the streptomycetes in this survey contained enzymes like that of *S. venezuelae* (A6) and *S. refuineus* (?), i.e., inhibited 100% by phenylalanine. Nothing has been reported on the transamination of phenylpyruvate to phenylalanine in streptomycetes.

4.1.1.3. Tyrosine. The synthesis of tyrosine in all streptomycetes, but one, precedes via arogenate, as in the pseudomonads, rather than via 4-hydroxyphenylpyruvate, as in enteric bacteria (Keller *et al.*, 1983). In other words, transamination precedes oxidation in streptomycetes and pseudomonads, and vice versa in enteric bacteria. This explained the inability of Lowe and Westlake (1971) to find prephenate dehydrogenase in *S. venezuelae* (A6). The one exception was a report of low levels of glutamine-dependent α-hydroxyphenylpyruvate aminotransferase activity and of phenylalanine stimulated prephenate dehydrogenase in *S. refuineus* (?) (Speedie and Park, 1980). These authors also confirmed the report of Chandra and Vining (1968) of the absence of a phenylalanine hydroxylase from streptomycetes, which explains why phenylalanine cannot be converted into tyrosine.

I have been unable to find any reports of arogenate transaminase, the first enzyme of tyrosine biosynthesis (Fig. 6) in streptomycetes. Keller *et al.* (1983) reported a number of differences in arogenate dehydrogenase in different streptomycetes (Table 15). The universally preferred cofactor was NAD^+. However, in *S. arenae* (A18), *S. gradiae* (?), *S. lavendulae* (F61) and *S. parvulus* (A12), $NADP^+$ was able to substitute, to some extent. *S. aureofaciens* (A14) was a special case because the enzyme in three of the seven strains tested could accept $NADP^+$ as a substitute for NAD^+, whilst that from the other four could not. Keller *et al.* (1985) reported the purification of arogenate dehydrogenase from *S. phaeochromogenes* (A40). The enzyme activity resided in a different polypeptide to chorismate mutase. This is different from the situation in enteric bacteria where there are two chorismate mutase isozymes, one of which is part of a polypeptide that includes the prephenate dehydratase activity, whilst the other is part of a polypeptide that includes the prephenate dehydrogenase.

The arogenate dehydrogenases from all the streptomycetes tested were insensitive to phenylalanine. Only the enzyme from *S. lavendulae* (F61) was insensitive to tyrosine; the enzymes from all the other streptomycetes were sensitive to differing degrees. Nothing was reported about regulation at the gene

level. However, Cushing *et al.* (1988), in a preliminary report, stated that they had isolated a tyrosine overproducing mutant of *S. niveus* (A1B) via selection of m-fluorotyrosine resistance, which might imply some regulation (see section 4.4).

4.1.1.4. Tryptophan. The organization of the genes for tryptophan biosynthesis has been examined in detail in *S. coelicolor* A3(2) (A21) (Engel and Watkins, 1974; Smithers and Engel, 1974; Hu *et al.*, 1999) and *S. venezuelae* (A6) (Doull *et al.*, 1986; Paradkar *et al.*, 1991b, 1993). In both cases, two clusters were found: one cluster encoded the early enzymes, anthranilate phosphoribosyl transferase (*trpD*, in *E. coli*) and phosphoribosyl anthranilate isomerase (*trpF*, part of *trpC* in *E. coli*); the other cluster encoded the late enzymes, indoleglycerol phosphate synthetase (*trpC*) and tryptophan synthase subunits α (*trpA*) and β and (*trpB*).

Rivero-Lezcano *et al.* (1990) cloned two fragments of *S. griseus* (A1B) DNA, one that complemented *E. coli trpA trpB*, and another that complemented *E. coli trpA trpB trpC* mutants. In each case the genes were transcribed from the pBR322 *tet* promoter and not the endogenous streptomycete promoter(s). Surprisingly, neither fragment showed any cross-hybridization with *S. lividans* (A21) DNA. *S. coelicolor* A3(2) (A21) DNA that complemented a *S. coelicolor* A3(2) (A21) *trpB* mutation was isolated and used to isolate a 21.4 kb DNA fragment that complemented *S. coelicolor* A3(2) (A21) *trpA* and *trpC* mutants but not *trpD* mutants. A 9.1 kb fragment was isolated that complemented a *trpD* mutation. DNA sequencing of part of the 21.4 kb fragment revealed three genes with strong amino acid sequence homology to *trpC*, *trpB* and *trpA* and arranged in that order (AF054585). The latter two genes were potentially translationally-coupled and the space between *trpC* and *trpB* contains a small ORF, *trpX*. It is very unusual for *trpF* not to be either fused to *trpC* or positioned downstream of it.

The sequencing of part of the 9.1 kb fragment (AF052077) revealed a *trpD* gene but no evidence of a *trpF* gene (Hu *et al.*, 1999). Complete sequencing of 36 734 bp of DNA around the *trpD* gene from the genome-sequencing project (AL049497) failed to identify a *trpF* gene. It may be relevant that a *trpF* gene was not identified in the *Mycobacterium tuberculosis* complete genome sequence (Hu *et al.*, 1999). Possibly the *trpF* of actinomycetes may be of a different class to other bacterial *trpF* genes as in DAHP synthase and 3-dehydroquinolase (section 4.1.1.1).

There are no reports of the isolation of mutants affected in anthranilate synthetase (*trpE* in *E. coli*). Paradkar *et al.* (1991a) reported the cloning of the anthranilate synthetase genes *trpE* and *trpG* of *S. venezuelae* (A6) by complementation of *E. coli trpE* and *trpG* mutants. The *trpG* gene is fused to *trpD* in *E. coli* but is independent in *Serratia marcescens* and encodes a glutamine amidotransferase. The *trpE* and *trpG* genes proved to be fused in the

streptomycete, with the *trpG* portion at the 3′ terminus (Lin *et al.*, 1998). Surprisingly, Francis *et al.* (1978) had reported the presence of a free, small subunit with glutamine amidotransferase activity associated with anthranilate synthase in *S. venezuelae* (A6).

Paradkar *et al.* (1993) reported the mapping of a thiostrepton-resistant marker that had been inserted at the site of the *trpEG* genes in *S. venezuelae* (A6). This marker mapped in the same segment of the chromosome as the *trpABC* gene cluster, but the two loci were distinct. Disruption of the *S. venezuelae* (A6) *trpEG* gene did not generate a Trp^- phenotype, implying that another copy of the gene was present (Lin *et al.* 1998).

The *S. coelicolor* A3(2) genome sequencing project has already turned up three *trpE* genes (http://www.sanger.ac.uk/Projects/s_coelicolor/). The *trpE1* gene (AL109661), which is a *trpEG* fusion, had already been isolated using the *S. venezuelae trpEG* gene and sequenced and proved to be a homologue of the Paradkar *et al.* gene (Hu, 1995). The *trpE2* gene (AL035654) was part of a cluster of tryptophan and aromatic genes apparently arranged in an operon. The gene order was: unknown, *trpE2 trpG2 trpD2 trpC2 aroH*. The unknown ORF, *trpE2* and *trpG2*, were potentially translationally-coupled, as were *trpD2 trpC2 aroH*. It is likely that the six genes form an operon as *trpG2* and *trpD2* are separated by only 16 base pairs. These genes are obviously silent as *trpC* and *trpD* Trp^- mutants were isolatable and complemented by the *trpCXBA* and *trpD* genes. The *unk–trpE2G2D2C2–aroH* cluster was associated with the region that encodes the calcium-dependent antibiotic. This lipopeptide ionophore contains two tryptophan residues, one L-form, the other L-form, which raises the possibility that the *unk–trpE2G2D2C2–aroH* cluster is exclusively involved in secondary metabolism. The gene *trpE3* (AL096884) did not have an associated *trpG* gene and proved to be separated from the *trpCXBA* cluster by only three genes.

Anthranilate synthetase of *S. venezuelae* (A6) showed evidence of tryptophan inhibition of enzyme activity and, to a lesser extent, anthranilate inhibition (Table 15). The apparent repression of gene expression was so small that its significance might be questioned. However, the level of repression seen was quite respectable, almost ten-fold, when a *trpC* mutant was starved of tryptophan (Lin *et al.*, 1998). A similar situation was seen in *S. parvulus* (A12) (Table 15).

Upstream of the *trpEG* gene was a region similar to an attenuation site. There was a small ORF, *trpL*, that encoded a small peptide with a tryptophan triplet, and the leader mRNA could potentially form two alternate configurations. This is the same configuration as in the *E. coli trp* attenuator. It was proposed that if there were sufficient tryptophan in the cell the TrpL peptide would be made and the leader mRNA could form two hairpin structures, the most 3′ of which would be a transcription terminator. Hence transcription of *trpEG* would cease. If tryptophan were limiting, however, the ribosome would

pause during TrpL translation; the two hairpins could not form because part of the mRNA would be sequestered by the ribosome. The formation of an anti-terminator hairpin would be encouraged and hence termination of *trpEG* transcription would not occur (Lin *et al.*, 1998). This was the first report of potential attenuation in streptomycetes. However, note the very recent observations of Craster *et al.* (1999) in which an apparent attenuation site in the branched-chain amino acids genes proved misleading (see section 4.1.4.3). The *trpEG1* gene of *S. coelicolor* A3(2) (A21) proved to have a very similar attenuator region, including a *trpL*, to that of *trpEG* of *S. venezuelae* (A6) (Hu, 1995). The *trpE2* (AL035654) and *trpE3* (AL096884) of *S. coelicolor* A3(2) (A21) had nothing that looked like attenuator sites in front of them.

Two promoters were identified for the *trpCXBA* cluster, one upstream of *trpC* and one upstream of *trpX*. One promoter was identified upstream of *trpD*. There was no evidence of tryptophan repression of any of these promoters, but they did appear to be growth-phase-dependent and growth-rate-dependent. The faster the growth rate, the greater the expression. The clear implication is that tryptophan biosynthesis was not regulated by product feedback repression but rather by the general growth requirements of the cell (Hu *et al.*, 1999).

4.1.1.5. p-Aminobenzoate. *p*-Aminobenzoate (pABA) is a precursor of tetrahydrofolate (Fig. 6) and candicidin, a secondary metabolite produced by *S. griseus* (A15) and fungimycin by *S. aminophilus* (A16). Gil *et al.* (1985) could find evidence only of pABA synthetase activity in these streptomycetes, and Johanni *et al.* (1989) reported that chorismate, not iso-chorismate, was the substrate of pABA synthetase. The activity was present only during the antibiotic production phase and was not subject to inhibition by the product or by anthranilate, aromatic amino acids or phosphate. Production of the enzyme was repressed 2.7-fold by tyrosine, eight-fold by phenylalanine, six-fold by tryptophan, 1.5-fold by pABA and 4.9-fold by 10 mM phosphate. No repression by anthranilate was seen (Gil *et al.*, 1985). The cloned pABA synthetase gene (Gil and Hopwood, 1983) proved to encode an enzyme in which the two subunits were fused (*pabAB*) and was associated with the candicidin biosynthetic cluster (Criado *et al.*, 1993). Phosphate regulated *pabAB* promoter activity (Rebollo *et al.*, 1989).

An intermediate in pABA synthesis is also required for chloramphenicol biosynthesis in *S. venezuelae* (A6). A pABA synthase gene was cloned from this streptomycete and proved to be a fused *pabAB* gene as in *S. griseus* (A15). Disruption of the gene led to reduced pABA synthesis, as assayed by sulphonamide sensitivity, and reduced chloramphenicol production. The isolation of Pab$^-$ mutants that still produced chloramphenicol implied that there are two sets of *pab* genes in *S. venezuelae* and that the cloned *pabAB* gene was involved in chloramphenicol biosynthesis (Brown *et al.*, 1996).

pABA synthetase activity could not be detected during the non-production

phase of growth of *S. griseus* IMRU 3570 (A1B) and *S. aminophilus* (A16), in mutants that did not produce the antibiotics and in streptomycetes that did not produce polyene macrolides; i.e. *S. coelicolor* A3(2) (A21), *S. lividans* (A21) and *S. griseus* NRRL 3851 (A1B). Yet all of these streptomycetes could grow in the absence of a tetrahydrofolate supplement and so they must have the enzyme. This was confirmed by the isolation of pABA auxotrophs of *S. coelicolor* A3(2) (A21) and *S. lividans* (A21) (Gil and Hopwood, 1983) and the cloning of *pabA* and *pabB* genes from *S. lividans* (A21) (Arhin and Vining, 1993). The implication is that the enzyme assay was too insensitive.

4.1.2. Histidine

Histidine biosynthesis requires ten enzyme activities. In enteric bacteria the genes of biosynthesis are clustered in a single region on the chromosome, whilst in *S. coelicolor* A3(2) (A21) there appears to be one big cluster at the '12 o'clock' position on the genetic map (*hisABCFGI*) and the two other genes at 'two o'clock' (*hisE*) and 'five o'clock' (*hisD*). DNA sequencing work led to the renaming of the genes in line with standard nomenclature (Limauro *et al.*, 1990). Of the 12 o'clock cluster, *hisA* was renamed *hisD*, *hisG* became *hisC*, *hisB* became *hisBd*, *hisC* became *hisH* and *hisA*. The five o'clock gene, *hisD*, was renamed *hisBp*. The two o'clock *hisE* gene appeared to be the *hisI* gene. None of the open reading frames encoded more than one enzymatic function. Two open reading frames have been identified to which no function has been ascribed.

The genes are transcribed in the order *hisD hisC hisBd orf1 hisH hisA* and *orf2*. Translational coupling was observed between *hisD/hisC*, *hisC/hisBd* and *orf1/hisH*. The *hisD* gene proved to be translated from a leaderless mRNA; i.e. the transcription initiation nucleotide, G, was the first G of the initiation codon GUG (Limauro *et al.*, 1992). The translational coupling provides circumstantial evidence for the presence of an operon. Carere *et al.* (1973) and Russi *et al.* (1973) proposed that the 12 o'clock cluster formed such an operon. The genetic evidence included the isolation of a *cis*-dominant constitutive mutant (*hisC119-O^c*) whose phenotype was consistent with an operator mutant. Enzyme assays revealed coordinate expression. Histidine plus histidinol repressed both genes of the 12 o'clock cluster tested (*hisD* and *hisBd*) 2.5- to 2.7-fold, whilst the 5 o'clock gene (*hisBp*) was repressed five-fold. The *hisC119-O^c* mutant abolished repression only of the 12 o'clock genes. These levels of repression are tiny when compared with those of other bacteria.

A mutant (RF-59) of *S. coelicolor* A3(2) secretes histidine (Derkos-Sojak *et al.*, 1977, 1985). The mutant was isolated as a revertant of a *hisB* mutant (presumably *hisBd*). It was shown to have the same sensitivity as wild-type *S. coelicolor* A3(2) to three histidine analogues but to be more resistant to another

analogue, 1,2,4-triazolealanine. Enzyme assays were developed for phosphoribosyl-ATP-pyrophosphatase (*hisG*), the first enzyme of histidine biosynthesis, D-erythroimidazoleglycerol phosphate dehydrase (*hisBd*) and histidinol dehydrogenase (*hisD*). In RF-59 the pyrophosphatase-specific activity was 2.9-fold higher, and the dehydrase- and dehydrogenase-specific activities were 4.7 to 4.9 higher than the wild type, when both strains were grown in minimal salts medium. The pyrophosphatase was inhibited completely by 10 mM histidine and 60% by 1 mM histidine. This property of the enzyme was not significantly affected in RF-59.

The lesion in RF-59 was mapped and found to lie between *argA* and *cysD* but closer to the former. This means the mutation cannot lie in the 12 o'clock cluster but could conceivably map close to *hisI* (formerly *hisE*). It has been shown in *Salmonella typhimurium* that mutations in the histidine tRNA genes or the histidinyl-tRNA synthetase can lead to derepression of the *his* operon, which led Derkos-Sojak *et al.* (1985) to propose a similar lesion in RF-59.

4.1.3. The Glutamate Family of Amino Acids

There are four members of the glutamate family of amino acids: glutamate, glutamine, arginine and proline. The biosynthesis of glutamate and glutamine are discussed in section 3.6.

4.1.3.1. Proline. Little has been reported concerning the biosynthesis of proline in streptomycetes. Hood *et al.* (1992) revealed that proline biosynthesis in *S. coelicolor* A3(2) (A21) occurs from glutamate via the intermediates γ-glutamyl phosphate, glutamate-γ-semialdehyde, and pyrroline-5-carboxylate (P5C). The interconversion of the last two compounds is a spontaneous Schiff's reaction. The enzymes involved in the pathway are γ-glutamate kinase, glutamate-γ-semialdehyde dehydrogenase and P5C reductase. The genes for the first two enzymes, *proB* and *proA* respectively, were cloned by complementation of the *proA1* mutation and sequenced. The sequence revealed the genes to be in the same order as those of *E. coli* but to be separated by an ORF of unknown function (*proX*). The *proC* gene was identified in the genome sequencing project (AL049819) and found to contain a peptide sequence obtained from the purified P5C reductase (D.D.S. Smith and D.A. Hodgson, unpublished).

Promoter probe studies revealed that both *proA* and *proB* have independent promoters that were constitutive. They did not appear to be significantly repressed by proline; indeed, there was a consistent two-fold induction by proline. The product of the *proC* gene (pyrroline 5-carboxylate reductase) was assayed and found to be constitutive and stimulated two-fold by addition of proline to the growth medium (Hood *et al.*, 1992).

4.1.3.2. Arginine. Arginine is the precursor of a number of important secondary metabolites of streptomycetes, such as streptomycin, sinefungin, clavulenic acid and azomycin. The early steps of arginine biosynthesis proceed from glutamate via a mechanism akin to that of proline biosynthesis, the difference being that the glutamate is first acetylated. The acetylation step ensures that the intermediates of arginine biosynthesis do not cyclize spontaneously to form the intermediates of proline metabolism.

Eight enzymes are involved in the arginine biosynthetic pathway. Udaka (1966) reported the presence of low levels of: *N*-acetylglutamate kinase, the second enzyme of the pathway (*argB*); ornithine carbamoyltransferase (OTCase), the sixth enzyme (*argF*); *N*-acetylornithase, the fifth enzyme (*argE*); and ornithine:acetylglutamate acetyltransferase, an alternative fifth enzyme and first enzyme (*argJ*), in *S. griseus* (A1B). There appeared to be two mechanisms for deactylation of the *N*-acetylornithine to produce ornithine. The transacetylase function reuses the acetate released from *N*-acetylornithine to acetylate glutamate. *N*-acetylornithase was the only enzyme found in enteric bacteria and bacilli. The transacetylase was found in fungi, pseudomonads and actinobacteria as well as the streptomycete. The transacetylase was also found in *S. coelicolor* A3(2) (A21) (Padilla *et al.*, 1991; Hindle *et. al.*, 1994). Ludovice *et al.* (1992) reported the cloning of an *E. coli argE*-complementing gene from *S. clavuligerus* (J71), which they called *argE*. However, the *S. coelicolor* A3(2) (A21) *argJ* gene also complemented an *E. coli argE* mutant (Hindle *et al.*, 1994). The *S. coelicolor* A3(2) *argJ* was part of an operon, *argCJB*. Upstream of *argC* (encoding *N*-acetylglutamyl-phosphate reductase) was a sequence similar to the major housekeeping sigma factor promoter consensus. Overlapping this potential promoter was a sequence similar to that of Arg boxes of *E. coli* and *B. subtilis* at which the cognate Arg regulators bind to activate arginine catabolism or repress arginine biosynthesis.

Evidence was also presented that *argH* (argininosuccinase; ASase), the eighth enzyme of the pathway, was linked to the *argCJB* operon, though separated by several kilobases. A number of the *S. coelicolor* A3(2) (A21) gene designations will have to be changed as the *argB2* mutation affects the *argH* gene (Hindle, 1990). The *argG* gene (argininosuccinate synthetase; ASS), the seventh enzyme of the pathway, is not linked to the *argCJB-H* cluster (Redenbach *et al.*, 1996).

Meade (1985) cloned the gene for ASS (*argG*) from *S. cattleya* (C47) using an *argG* auxotroph of *E. coli* as recipient. The gene was localized to a 1.5 kb fragment and appeared to be expressed in *E. coli* by a promoter within the fragment. Similar cross-genus transcription was seen with the *S. coelicolor* A3(2) (A21) *argCJB* operon (Hindle *et al.*, 1994) and *S. clavuligerus* (J71) (Ludovice *et al.*, 1992). When the *argG* gene was used to probe the Arg^- aerial mycelium$^-$ (Amy^-) mutants, which had previously been shown not to revert, it was clear that all of them had deleted the gene. Indeed, a large portion of the chromosome had

been lost in these mutants. However, when just the *argG*$^+$ gene was returned to these strains, they lost both the Arg$^-$ and the Amy$^-$ phenotypes concomitantly. The *argG* genes of *S. coelicolor* A3(2) (A21) and *S. lividans* (A21) were cloned at the same time as that of *S. cattleya* (C47) by complementation of *argG* mutants of the two streptomycetes. These genes were also used to confirm that *argG* mutants contained large deletions (Ishihara *et al.*, 1985). The *S. lavendulae* (F61) *argG* gene was similar to that of *S. cattleya* (C47), *S. lividans* (A21) and *S. coelicolor* A3(2) (A21) and appeared to be translated from a leaderless mRNA (Ogawara *et al.*, 1993). The *argG* gene of *S. clavuligerus* (J71) was cloned by complementation of *E. coli* and *S. lividans* (A21) *argG* mutants. The gene product proved to have a very different structure from the streptomycete ASSs and looked more like methanotroph ASSs. The *argH* gene was located 3′ to the *argG* gene in *S. clavuligerus* (J71) (Roderiguez-Garcia *et al.*, 1995).

Vargha *et al.* (1983) reported an Arg$^-$ Amy$^-$ mutant of *S. fradiae* (G68), but in this case the lesion was in the OTCase gene (*argFI*). Szilagyi *et al.* (1987) purified the enzyme and examined its properties but did not report its regulation. From these studies and others it has been suggested that citrulline is involved in the initiation of secondary metabolism and morphological differentiation of streptomycetes (Ochi *et al.*, 1984).

Some information has been reported on the nature and type of regulation of the arginine biosynthetic genes in streptomycetes. Udaka (1966) reported that in *S. griseus* (A1B) the *N*-acetylglutamate kinase (*argB*) could be inhibited by 70–100% by 5 mM arginine, and the OTCase gene (*argFI*) was repressed one- to two-fold by arginine. Padilla *et al.* (1991) reported that this same enzyme in Arg$^+$ *S. coelicolor* A3(2) (A21), *S. lividans* (A21), *S. griseus* (A1B) and *S. clavuligerus* (J71) cells was repressed, 1.5-fold, 3.4-fold, 2.4-fold and 2.3-fold, respectively, by 0.5 mM arginine. When the *argC* gene of *S. clavuligerus* (J71) was carried on a multicopy plasmid in *S. lividans* (A21), the *N*-acetylglutamate-phosphoreductase activity became assayable for the first time. Addition of 25 mM arginine repressed expression of the gene two-fold (Ludovice *et al.*, 1992).

Flett *et al.* (1987) reported the activity levels of OTCase (*argFI*), ASS (*argG*) and ASase (*argH*) in wild-type and *argFI* and *argG* mutants of *S. coelicolor* A3(2) when grown in low (10 $\mu g\ ml^{-1}$) arginine media. In the *argFI* mutant the levels of ASS and ASase were derepressed 37.5-fold and 1.9-fold, respectively, relative to the wild-type levels. In the *argG* mutants the levels of activity with respect to the wild type were between 14.2- and 47.1-fold higher for the OCTase and between 2.1- and 7.4-fold higher for the ASase. Not only were the two enzymes derepressed to different levels, but also, in different mutants, the ratio of derepression of the two enzymes was not the same. It is clear, therefore, that a simple coordinated model of repression of the arginine biosynthetic genes by arginine is not easily envisaged.

As stated above, Hindle *et al.* (1994) reported the presence of potential

Arg boxes overlapping the putative *argCJB* promoter in *S. coelicolor* A3(2) (A21). When the *argCJB* promoter region was inserted in multicopy in *S. coelicolor* A3(2) there was a transient derepression of *argFI* as measured by OTC assays. This region also specifically bound *in vitro* the AhrC arginine regulator protein of *B. subtilis*. These two observations imply an arginine regulatory system similar to that seen in *E. coli* and *B. subtilis* (Soutar and Baumberg, 1996).

Similar experiments using the *argC* promoter in multicopy in *S. clavuligerus* (J71) and *in vitro* footprinting with the *B. subtilis*, AhrC and the *S. clavuligerus* (J71) *argC* agreed with the *S. coelicolor* A3(2) results (Rodriguez-Garcia *et al.*, 1997). However, these authors also found a gene, *argR*, 5′ of *argG* that in multicopy showed lower OTC (*argFI*) activity and increased activity of OAT, an enzyme associated with arginine catabolism by this group (section 3.3.3.2). This is the expected phenotype if ArgR is a repressor of arginine biosynthesis and an activator of arginine catabolism. There was no report of the phenotype of any mutants of *argR*.

4.1.4. The Branched-Chain Amino Acids

A great deal of interest has centred on the metabolism of the branched-chain amino acids (valine, isoleucine and leucine) in the streptomycetes. This is because the biosynthesis and catabolism of these amino acids proceeds via intermediates that are precursors to a wide range of polyketide and polyether antibiotics (Reynolds *et al.*, 1988) (section 3.3.4).

4.1.4.1. Isoleucine and Valine. The biosynthesis of isoleucine and valine proceeds via an enzyme pathway that involves the same enzymes for both amino acids but uses different precursors. The precursor of valine is α-acetolactate, which is the product of the condensation of two pyruvate molecules by acetohydroxy acid synthase (AHS). The same enzyme also catalyses the condensation of pyruvate and α-ketobutyrate to form α-aceto-α-hydroxybutyrate, the precursor of isoleucine, and is a product of the *ilvBN* genes. In enteric bacteria there are three isozymes of AHS each with different regulatory properties. α-ketobutyrate is derived by deamination of threonine by the enzyme threonine dehydratase (*ilvE*). The other enzymes of the pathway are: acetohydroxy acid isomeroreductase (AHIR; *ilvC*); dihydroxy acid dehydrase (DHAH; *ilvD*); and a transaminase (*ilvE*). In enteric bacteria a number of transaminases are present. One enzyme, transaminase B, is capable of transamination of precursors to form valine, isoleucine and leucine. Transaminase C is used to form valine alone.

Threonine dehydratase (TD) is amphibolic (i.e. it may be involved in the catabolism of threonine and the biosynthesis of isoleucine) and is encoded by

the *ilvA* gene. In *S. fradiae* (G68), threonine appeared to be catabolized via threonine aldolase (section 3.3.5.3), whilst TD was involved in the control of isoleucine synthesis (Vancura *et al.*, 1988b). Vancura *et al.* (1988b, 1989a) reported that TD was induced 2.5-fold by valine, 6.6-fold by valine plus isoleucine, 1.4 to 2.0-fold by threonine, and 1.4-fold by glutamate, but repressed 1.6-fold by isoleucine. No regulation of enzyme activity by valine, leucine or isoleucine could be observed using the amino acids at 10 mM. However, 200 mM ammonium caused >66% inhibition. Surprisingly the affinity of the enzyme for its substrate was very low; i.e. the K_m for threonine was 230 mM. Bascaran *et al.* (1989a) reported that the TD of *S. clavuligerus* (J71) was not inhibited by isoleucine; this led them to assume that the enzyme had a catabolic role, which, considering the data from *S. fradiae* (G68), might be an unwarranted assumption. They also reported that ammonium induced the activity of TD 2.3 to 3.3-fold. They did not report the effects of isoleucine or valine in the medium.

AHS was assayed in *S. fradiae* (G68) (Vancura *et al.*, 1989a) and found to be insensitive to any of the branched-chain amino acids. The enzyme was induced 2.1-fold by valine, 2.5-fold by valine plus leucine, and 2.4-fold by all three branched-chain amino acids together. The enzyme was repressed two-fold by isoleucine and 1.2-fold by leucine. Preliminary fractionation experiments implied that only one isozyme of AHS was present in these cells. The *ilvB*, *ilvN* and *ilvC* cluster was cloned from *S. avermitilis* (?). The AHS large and small subunits (products of *ilvB* and *ilvN*, respectively) were expressed in *E. coli* and shown to complement the equivalent mutant and to be inhibited 50–75% by 100 μM valine (De Rossi *et al.*, 1995).

Vancura *et al.* (1988b, 1989a) assayed a branched-chain amino acid transaminase in *S. fradiae* (G68). It was impossible to tell whether there was more than one type of transaminase present. The presence of ammonium, glutamate and threonine in the growth medium had no effect on enzyme levels except when glutamate and threonine were present together, when enzyme activity increased two-fold. Valine induced the enzyme 2.9-fold, leucine eight-fold, and isoleucine 15.3-fold. All three amino acids present at the same time induced the enzyme 18-fold, which may imply a catabolic role.

4.1.4.2. Leucine. Leucine is synthesized via the isopropylmalate pathway. The first intermediate of this pathway is the penultimate product of the valine pathway: α-ketoisovalerate. The pathway makes use of the following enzymes: isopropyl malate synthase (IPMS; *leuA*); isopropyl malate isomerase (ISOM; *leuCD*); β-isopropyl malate dehydrogenase (IPMD; *leuB*); and an aminotransferase (*ilvE*) (section 4.1.4.1). Evidence was presented by Martin *et al.* (1962) that a key intermediate of this pathway, β-carboxy-β-hydroxyisocaproic acid (β-isopropylmalate), was recoverable from the culture filtrates of three actinomycelates, *S. erythreus* (now *Saccharopolyspora erythraea*),

Nocardia lurida and a *Corynebacterium* sp., which implied that this pathway is common to the actinomycelates.

Hercomb *et al.* (1987) reported the cloning of *leuA* of *S. rochei* (A12) by complementation of an *E. coli leuA* mutation. The *leuA*-complementing fragment did not include any *leuB* complementing activity. Stieglitz and Calvo (1974) reported the presence of IPMD activity, the third enzyme of the leucine pathway, in *S. violaceoruber* (A21). However, they did not report any regulatory properties of the enzyme. These authors did report that *Nocardia opaca* contained an IPMS activity that was inhibited 90% by 0.25 mM leucine.

4.1.4.3. Regulation of the Branched-Chain Amino Acids. The specific activities of first enzymes of the valine–isoleucine and leucine pathways, AHS (*ilvBN*) and IPMS (*leuA*), were assayed in *S. coelicolor* A3(2) (A21) cells grown in minimal salts medium with and without various combinations of branched-chain amino acids. Isoleucine stimulated expression of both genes two- to three-fold. Isoleucine plus valine plus leucine repressed the genes five- to eight-fold, implying feedback repression (Potter and Baumberg, 1996). As confirmation of feedback repression, it proved possible to isolate branched-chain amino acid-analogue resistant mutants that were derepressed for *leuA* and *ilvBN* expression together or singly in *S. coelicolor* A3(2) (A21) (Potter and Baumberg, 1996) or overproduced α-ketoisovalerate owing to deregulation of the biosynthetic pathway, as assayed by AHS activity in *S. cinnamonensis* (?) (Pospisil *et al.*, 1998). Interestingly, all the *S. cinnamonensis* mutants also overproduced VDH, presumably because valine was overproduced and hence the catabolic pathway (section 3.3.4.1) was induced.

When the *ilvBNC* and *leuA* genes of *S. coelicolor* A3(2) (A21) were sequenced, it appeared that there were structures typically seen in bacterial attenuation sites as previously seen for the *S. venezuelae* (A6) and *S. coelicolor* A3(2) (A21) *trpEG1* genes (section 4.1.1.4). No such attenuation structures were seen upstream of *ilvD*, *ilvE*, *leuB* or *leuCD*. However, when the *ilvBNC*, *ilvD*, *leuA*, *leuB* and *leuCD* promoters were inserted into a promoter probe, the promoters proved to be repressible by a mixture of all three branched-chain amino acids. When the *ilvBNC* and *leuA* leader peptides were mutated to remove the branched-chain amino acid residues, both genes remained repressible by branched-chain amino acids. From this it was concluded there was a mechanism of repression of all branched-chain amino acid genes but that attenuation was not important (Craster *et al.*, 1999).

4.1.5. The Aspartate Family of Amino Acids

The aspartate family of amino acids includes aspartate, asparagine, lysine, threonine and methionine. (Methionine is discussed in section 4.1.8.) Very

little work has been reported on aspartate and asparagine biosynthesis. As discussed above (sections 3.3.5.1 and 3.6.4), one enzyme of aspartate metabolism, glutamate:oxaloacetate transaminase (GOAT) (Fig. 3), was reported to be present in cell free extracts of *S. coelicolor* A3(2) (A21) (Fisher, 1989) and *S. noursei* (?) (Grafe *et al.*, 1974b). The *S. coelicolor* A3(2) (A21) enzyme appeared to be insensitive to the nature of the nitrogen source in the growth medium, as essentially the same specific activity was found in cells grown on complex medium, ammonium, aspartate, asparagine, glutamine or histidine.

4.1.5.1. Lysine. Much has been reported concerning the anabolic pathways of lysine in streptomycetes. This is because a number of important secondary metabolites are produced from lysine, including β-lactams and nourseothricin. The biosynthesis of lysine can occur by either the aminoadipate (AAA) pathway, which does not use aspartate as an intermediate, or the diaminopimelate (DAP) pathway, which does. Lingens and Vollprecht (1964) reported that *S. griseus* (A1B) used the DAP pathway. They also reported circumstantial evidence that *S. venezuelae* (A6) used the DAP pathway but that *S. antibioticus* (A31) used the AAA pathway. Kirkpatrick *et al.* (1973) reported that *S. lipmanii* (A7) used the DAP pathway. Sawada *et al.* (1977) reported the presence of the DAP pathway in *S. lavendulae* (F61). Their report was based on the observation that ^{14}C lysine accumulated intracellularly when ^{14}C DAP was added to the cell but not when ^{14}C AAA was used. The DAP pathway was also found in *S. clavuligerus* (J71) (Mendelovitz and Aharonowitz, 1982). The gene for aspartate semialdehyde dehydrogenase, *asd*, was cloned from *S. akiyoshiensis* (?) and it was not linked to the gene for aspartokinase. Aspartokinase and aspartate semialdehyde dehydrogenase are the first enzymes in the pathway that lead to DAP (Le *et al.*, 1996).

Regulation of lysine synthesis has been investigated in *S. lipmanii* (A7), *S. clavuligerus* (J71) and *S. noursei* (?). DAP accumulation was seen only in a mutant of *S. lipmanii* (A7) lacking DAP decarboxylase and not in the wild type. The accumulation of DAP by the mutant was decreased by the addition of lysine to the growth medium. In media containing limiting amounts of lysine, the mutant began to accumulate DAP, implying that the enzymes of DAP synthesis were repressed or inhibited by lysine. Addition of lysine to the wild type did not repress DAP carboxylase levels. However, lysine did inhibit the enzyme by as much as 66%. Addition of DAP to the wild type did not activate DAP carboxylase (Kirkpatrick *et al.*, 1973).

Mendelovitz and Aharonowitz (1982) investigated the regulation of a number of enzymes of the aspartate amino acid family pathways in *S. clavuligerus* (J71). Aspartyl phosphate, the product of aspartokinase, is a precursor of methionine, threonine (and hence isoleucine; see section 4.1.4.1) and lysine biosynthesis. In enteric bacteria there are three isozymes of

aspartokinase, each of which is regulated differently. However, the evidence from streptomycetes points to the possession of just one enzyme. The aspartokinase of *S. clavuligerus* (J71) was stimulated 1.4- to two-fold by the addition of lysine. The addition of methionine, threonine, isoleucine, homoserine, meso-DAP or AAA had no effect. Joint addition of lysine and threonine resulted in the 94% inhibition of the enzyme. The further addition of isoleucine to the lysine plus threonine decreased the level of inhibition to 85%. Substituting methionine for isoleucine decreased the inhibition to 81%. Isoleucine repressed enzyme formation by 70%. Methionine repressed the enzyme two-fold. Lysine activated enzyme synthesis 1.2-fold. The same general observations were made in *S. fradiae* (G68) (Vargha, 1997). An attempt to purify the enzyme from *S. clavuligerus* (J71) was made and the K_m values for ATP (2.8 mM) and aspartate (17 mM) determined. Mutants were isolated in which the aspartokinase was insensitive to lysine plus threonine feedback inhibition (Mendelovitz and Aharonowitz, 1983). The lysine analogue S-(2-aminoethyl)-L-cysteine (AEC) was used, which could substitute for lysine in the concerted feedback inhibition with threonine. Most AEC-resistant mutants (70%) contained a mutant aspartokinase. The greater proportion (70%) of this subclass of mutants (50% of the total AEC-resistant mutants) produced a higher titre of the β-lactam antibiotic.

The first enzyme of the exclusive pathway of lysine biosynthesis, dihydrodipicolinic acid synthetase, was assayed and found to have a K_m for aspartate-semialdehyde of 0.3 mM and for pyruvate of 1 mM (Mendelovitz and Aharonowitz, 1982). The enzyme was inhibited by both DAP and AAA. Lysine had no effect. Lysine by itself also had no effect on enzyme biosynthesis. However, when supplied in the presence of threonine there was an activation of the enzyme 1.25-fold. DAP, leucine and alanine each caused a reduction in activity of the enzyme when present in the culture media. It should be noted that all these experiments were carried out on wild-type strains, so there is always the possibility that the amino acids present in the intracellular pools could be causing inhibition/stimulation or repression/activation. An AEC-resistant mutant with a mutated aspartokinase was also assessed for any effects on its dihydrodipicolinic acid synthetase; none was found (Mendelovitz and Aharonowitz, 1983).

The intracellular amino acid concentration of one of the AEC-resistant mutants was analysed in detail (Aharonowitz *et al.*, 1984). The levels of methionine were elevated (2.6- to 6.5-fold) in the mutant. The levels of threonine, lysine and all other amino acids were about the same. However, the DAP content of the cells was increased dramatically; i.e. some 40-fold. As meso-DAP and LL-DAP were found in about equal proportion, it implies that DAP epimerase, the penultimate enzyme of the DAP pathway, was fully active. This implied that aspartokinase and DAP decarboxylase were important control points of lysine production. Later work (Aharonowitz *et al.*,

1986) revealed that the latter enzyme can be inhibited by 49% with high (10 mM) levels of lysine or by 64% with alanine. This enzyme was also implicated in control of lysine synthesis in *S. lipmanii* (A7) (Kirkpatrick *et al.*, 1973).

The aspartokinase of *S. noursei* (?) was assayed in the presence and absence of lysine and threonine (Hanel *et al.*, 1985). Like the *S. clavuligerus* (J71) enzyme, it was stimulated by lysine alone (1.7-fold) and feedback inhibited by threonine plus lysine. In this case, though, the inhibition was merely 60%, compared with 94%. An AEC-resistant mutant was isolated and found to have higher nourseothricin (a β-lysine containing antibiotic) production capabilities. However, its aspartokinase was still subject to feedback inhibition; indeed, it was subject to more stringent repression (90%) and lysine no longer stimulated it, but there was greater (3.5-fold) enzyme activity. A couple of mutants with affected aspartokinase had been isolated by other methods. One, isolated as phosphate-deregulated, contained 5.2-fold more enzyme that was subject to the usual regulation. Another, isolated as an increased antibiotic producer, contained an enzyme resistant to feedback control. It appears, therefore, that what was true for *S. clavuligerus* (J71) was also true for *S. noursei* (?).

4.1.5.2. Threonine. Two enzymes are unique to threonine synthesis, homoserine kinase and threonine synthase. A further enzyme, homoserine dehydrogenase, is unique to both threonine and methionine biosynthesis. The latter enzyme has been assayed in *S. clavuligerus* (J71) and found to have a K_m for aspartate semialdehyde of 0.28 mM (Mendelovitz and Aharonowitz, 1982). Threonine inhibited the enzyme by 75% whilst homoserine inhibited it by a half and methionine by a fifth. Threonine repressed enzyme synthesis by 15% and isoleucine by 45%. Lysine, on the other hand, stimulated enzyme synthesis 1.2-fold.

4.1.6. The Pyruvate Family of Amino Acids

As discussed in section 3.3.6, alanine is synthesized by amination of pyruvate. This can occur by transamination or by alanine dehydrogenase-mediated condensation of ammonium with pyruvate generating oxidized NAD^+. As discussed in section 3.3.6, AOAT activity (Fig. 3) could be found in neither *S. coelicolor* A3(2) (A21) (Fisher, 1989) nor *S. clavuligerus* (J71) (Brana *et al.*, 1986a). However, it was found in *S. hygroscopicus* (A32) and *S. noursei* (?) (Grafe *et al.*, 1974a, b), *S. cyanogenus* (?) (Watanabe *et al.*, 1976a) and *S. avermitilis* (?) (Novak *et al.*, 1992a). Alanine:2-isovalerate transaminase could not be found in *S. coelicolor* A3(2) (A21) (Fisher, 1989). Those streptomycetes with AOAT could generate alanine from glutamate. However, a lot of streptomycetes contained only ADH (Fig. 3) and so presumably they must use the reverse reaction

of this enzyme, even though alanine often induced production of the enzyme, implying a catabolic role. It should be noted that in a number of streptomycetes ADH was induced also by ammonium, implying an anabolic role (section 3.3.6).

4.1.7. The Serine Family of Amino Acids

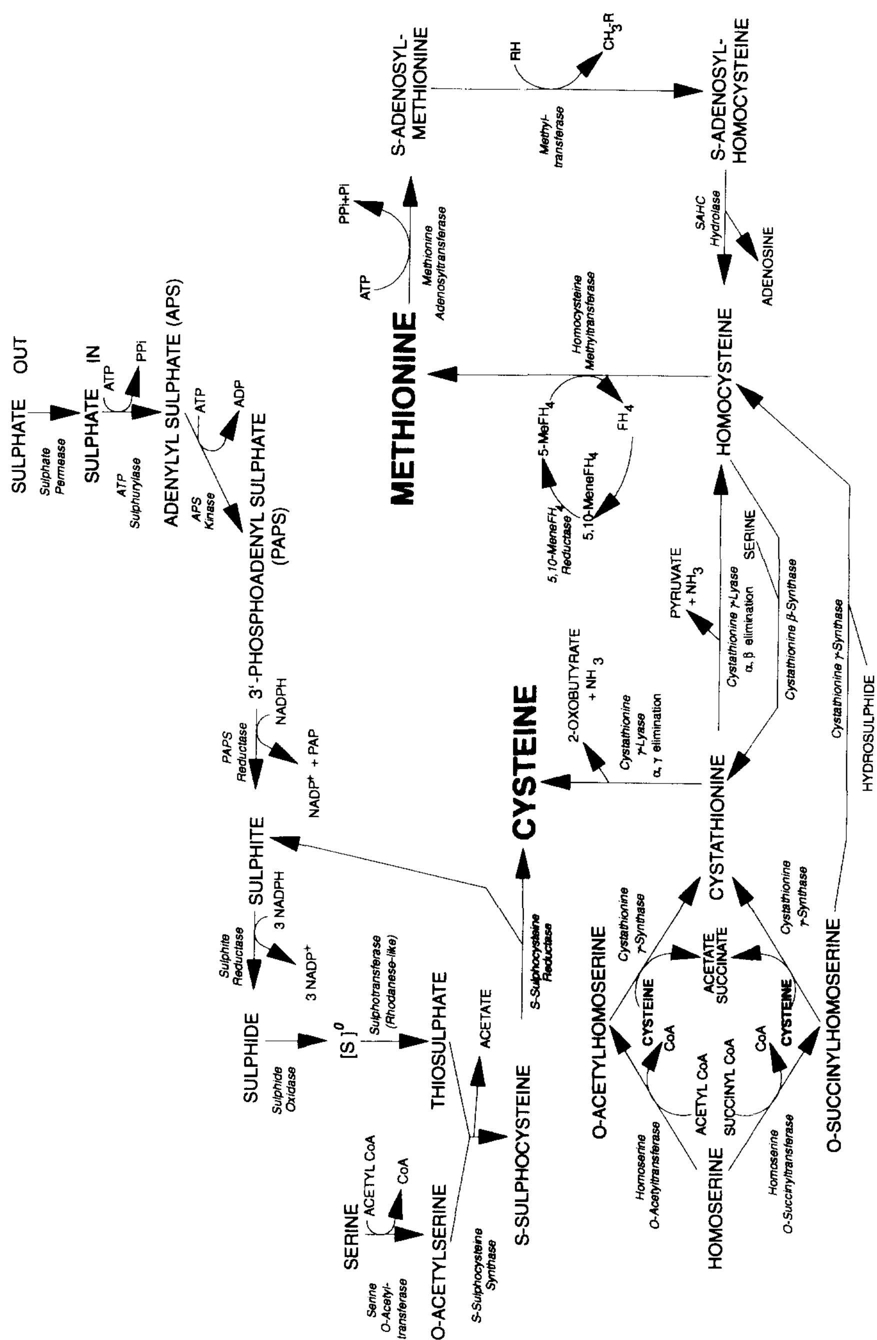
There are three members of the serine group of amino acids: serine, glycine and cysteine. The latter is discussed in section 4.1.8.

Parada (1981) reported that addition of serine to minimal medium inhibited the growth and germination of *S. aureofaciens* (A14) and *S. rimosus* (B42). This inhibition could be reversed by the addition of methionine and glycine. One explanation was that serine repressed or inhibited an enzyme or enzymes necessary for the biosynthesis of methionine and glycine. Glycine is synthesized from serine by the transfer of a hydroxymethyl group from serine to tetrahydrofolate. Methionine is synthesized by the transfer of a methyl group from methyl-tetrahydrofolate to homocysteine (see section 4.1.8.3). It is possible that serine affected folic acid metabolism in some way. Parada isolated a mutant of *S. aureofaciens* (A14) that could tolerate serine, but the mechanism of resistance was not clear. There was no change in the serine catabolic enzyme serine deaminase.

4.1.8. The Sulphur-Containing Amino Acids

I have chosen to discuss cysteine and methionine in a separate section because sulphur metabolism in streptomycetes is different from that in other bacteria. Streptomycetes possess the enzymes of the trans-sulphuration pathway previously thought to be found solely in eukaryotes. The pathway allows the transfer of sulphur from methionine to cysteine.

4.1.8.1. Cysteine. Cysteine biosynthesis in *S. griseus* subsp. *cryophilus* (A1B) is initiated by the import of sulphate, which is then subjected to a series of reduction steps to form sulphide. The final inorganic sulphur intermediate, thiosulphate, is then combined with O-acetylserine, the product of serine acetylation, to form S-sulphocysteine. This intermediate is reduced to cysteine and sulphite (Fig. 7). The proposal that cysteine biosynthesis in this streptomycete goes via thiosulphate, and not via the condensation of sulphide and O-acetylserine, comes from mutant analysis (Kitano *et al.*, 1985). This pathway is more like that seen in fungi. However, Nagasawa *et al.* (1984) reported the presence of an O-acetylserine sulphydrase activity in *S. phaeochromogenes* (A40). This is the enzyme capable of generating cysteine directly from condensation of O-acetylserine and sulphide that is present in enteric bacteria.



SULPHATE OUT
Sulphate Permease
SULPHATE IN
ATP
PPi
ATP Sulphurylase
ADENYLYL SULPHATE (APS)
ATP
ADP
APS Kinase
3'-PHOSPHOADENYL SULPHATE (PAPS)
PAPS Reductase
NADPH
NADP+ + PAP
SULPHITE
3 NADPH
Sulphite Reductase
3 NADP+
SULPHIDE
Sulphide Oxidase
[S]0
Sulphotransferase (Rhodanese-like)
THIOSULPHATE
ACETATE
SERINE
ACETYL CoA
CoA
Serine O-Acetyl-transferase
O-ACETYLSERINE
S-Sulphocysteine Synthase
S-SULPHOCYSTEINE
S-Sulphocysteine Reductase
CYSTEINE
2-OXOBUTYRATE + NH3
Cystathionine γ-Lyase α, γ elimination
METHIONINE
5-MeFH4
5,10-MeneFH4
5,10-MeneFH4 Reductase
FH4
Homocysteine Methyltransferase
ATP
PPi+Pi
Methionine Adenosyltransferase
S-ADENOSYL-METHIONINE
RH
CH3R
Methyl-transferase
S-ADENOSYL-HOMOCYSTEINE
SAHC Hydrolase
ADENOSINE
HOMOCYSTEINE
PYRUVATE + NH3
Cystathionine γ-Lyase α, β elimination
SERINE
Cystathionine β-Synthase
Cystathionine γ-Synthase
HYDROSULPHIDE
CYSTATHIONINE
O-ACETYLHOMOSERINE
Cystathionine γ-Synthase
CYSTEINE
ACETATE
SUCCINATE
CoA
ACETYL CoA
SUCCINYL CoA
Homoserine O-Acetyltransferase
HOMOSERINE
Homoserine O-Succinyltransferase
O-SUCCINYLHOMOSERINE

Sulphate transport was active and inhibited by sulphide, sulphite, thiosulphate and cysteine, but not by methionine, in *S. griseus* subsp. *cryophilus* (A1B). Cysteine repressed sulphate transport 50-fold but thiosulphate had only a mild repressive effect. Mutants blocked in transport could be isolated using selenate (Kitano *et al.*, 1985). The observation that thiosulphate was an intermediate in cysteine anabolism in at least one streptomycete may be related to the observation that streptomycetes can oxidize sulphur compounds to thiosulphate (Yagi *et al.*, 1971; Kunert and Stransky, 1988) (section 1.4).

Lydiate *et al.* (1988) isolated a series of selenate-resistant mutants of *S. coelicolor* A3(2) (A21), *S. lividans* (A21), *S. glaucescens* (A28), *S. olivaceus* (A1C), *S. pactum* subsp. *pactum* (C44) and *S. rimosus* (B42). The *S. coelicolor* A3(2) (A21) and *S. lividans* (A21) mutations fell into three classes. The *selC* class, which appeared to be the same as the previously identified *cysD* strains, coded for the sulphate transport system. The *selA* mutants appeared to be blocked in the first enzyme of the sulphate reduction pathway, ATP sulphurylase. The *selB* class appeared related to *cysC* mutants. DNA clones from *S. coelicolor* A3(2) (A21) were isolated that complemented a *selA* lesion. The overlapping DNAs and DNAs isolated from *S. clavuligerus* (J71) and *S. cattleya* (C47) were capable of complementing a number of the *sel* and *cys* mutations, implying that the genes were clustered and that they had not diverged significantly as the genus had developed. From a study of the complementation patterns a physical genetic map of the area was devised. It was also clear that a number of mutations having a complex phenotype (i.e. appearing to have lost a number of sulphur metabolite transport functions as well as sulphate transport) were due to DNA deletions.

Donadio *et al.* (1990) attempted to isolate the gene for a P-450 hydroxylase from *Saccharopolyspora erythraea*, previously *S. erythreus* (A1C), and developed an oligonucleotide from an *N*-terminal sequence derived from the purified protein. The DNA clones they obtained did not encode the P-450 hydroxylase, which was *N*-terminal blocked, but rather a rhodanese-like protein that had contaminated the P450 hydroxylase preparation. Rhodanese catalyses the transfer of a sulphane atom (sulphur with a neutral oxidation state) from thiosulphate ($S_2O_3^{2-}$) to a receptor with the concomitant release of sulphite (SO_3^{2-}). When the rhodanese open reading frame was disrupted in an otherwise wild-type strain, it developed a cysteine requirement. A cell extract from the mutant retained full rhodanese activity, so the one that had been destroyed was a minor component of the readily assayable activity. A model was presented showing the rhodanese-like gene acting as a transfer factor (sulphotransferase) of the product

Figure 7 Pathways of sulphur-containing amino acid biosynthesis in streptomycetes (see text for references). 5-MeFH$_4$ = 5-methyltetrahydrofolate; 5,10-MeneFH$_4$ = $N^{5,10}$-methylenetetrahydrofolate; PAP = 3′-phosphoadenosine 5′phosphate; CoA = co-enzyme A; RH = generic methyl acceptor.

of sulphide oxidase reaction which was condensed with sulphite to form thiosulphate (Fig. 7). Because two other open reading frames were identified closely linked to the rhodanese (separated by 29 bp and 2 bp), the authors proposed that they were also involved in sulphur metabolism. Upstream of the three open reading frames was a very short open reading frame of 14 amino acids, of which three were cysteines. It is tempting to speculate on a regulatory role for such a peptide, perhaps involving attenuation.

4.1.8.2. The Trans-sulphuration Pathway. The first indication of the potential for a trans-sulphuration pathway in streptomycetes came from the work of Whitney *et al.* (1972). These workers found that the ^{35}S label in methionine ended up in a *S. clavuligerus* (J71) β-lactam antibiotic that was derived from cysteine. This raised the possibility that there existed a pathway for the interconversion of methionine and cysteine. Kern and Inamine (1981) demonstrated the presence of cystathionine γ-lyase, an important enzyme of the trans-sulphuration pathway, in lysates of *S. lactamdurans* (A7) and *S. cattleya* (C47). This enzyme catalyses the cleavage of L-cystathionine into cysteine, α-ketobutyrate and ammonium – the α,γ-elimination reaction. The *S. lactamdurans* (A7) gene encoding the enzyme was found to be derepressed three-fold by growth in sulphate-limited media, and the enzyme was shown to be inhibited by cysteine, implying feedback regulation.

Nagasawa *et al.* (1984) reported the results of a survey of actinomycetes and other bacteria for the presence of cystathionine γ-lyase. All the actinomycetes tested – *Micromonospora* spp., *Micropolyspora* spp., *Mycobacterium* spp., *Nocardia* spp., *Streptosporangium* spp., *S. lactamdurans* (A7), *S. lipmanii* (A7), *S. lydicus* (A29), *S. olivochromogenes* (A19), *S. phaeochromogenes* (A40), *S. rimosus* (B42), *S. virginiae* (F61), and *Streptoverticillium kentuchense* (F single member cluster) – contained the enzyme, whilst the other bacteria (including the actinobacteria *Corynebacterium aquaticum*, *Arthrobacter* spp., *Brevibacterium* spp., and *Cellulomonas* spp.) did not.

The cystathionine γ-lyase (Fig. 7) from *S. phaeochromogenes* (A40) was purified and characterized. The enzyme was made up of four identical subunits and contained four molecules of pyridoxal 5′-phosphate. The enzyme was capable of the α,β-elimination reaction, i.e. cleavage of L-cystathionine into homocysteine, pyruvate and ammonium. The α,β-elimination reaction occurred with about one-seventh of the frequency of the α,γ-elimination reaction. The enzyme from *S. phaeochromogenes* (A40) was found to be serologically related to the cystathionine γ-lyases from all the streptomycetes, *Micromonospora* spp. and *Micropolyspora* sp. tested, but not to the enzyme from *Nocardia erythropolis* or *Mycobacterium smegmatis* (Nagasawa *et al.*, 1984). Further work (Kanzaki *et al.*, 1987) revealed that the enzyme has a regulatory site, responsible for L-cysteine binding and consequent feedback inhibition, and a split active site. The two subsites of the active site perform

different functions: subsite 1 binds pyridoxal 5′-phosphate and the L-amino acid moiety of cystathionine or homoserine; whilst subsite 2 has a relatively broad specificity for thiol compounds with a carboxyl group.

The other enzyme of trans-sulphuration pathway, cystathionine β-synthase, which converts homocysteine to cystathionine by the addition of serine (Fig. 7), was found in extracts of *S. phaeochromogenes* (A40). Therefore, *S. phaeochromogenes* (A40) has all the enzymes necessary for the trans-sulphuration pathway (Nagasawa *et al.*, 1984).

4.1.8.3. Methionine. Kirkpatrick and Godfrey (1973) made an interesting observation. They isolated *thr* mutants of *S. lipmanii* (A7) that could be stimulated by homoserine. Homoserine auxotrophs have been isolated in other bacteria before; however, in each case there was always a requirement for homoserine or threonine plus methionine, the genetic lesion being in homoserine dehydrogenase which is an enzyme of both the threonine pathway and the methionine pathway. In *S. coelicolor* A3(2) (A21), putative homoserine dehydrogenase mutants (*mthB*) require methionine plus threonine or homoserine (Hopwood *et al.*, 1973). It is difficult to explain the *S. lipmanii* (A7) anomaly unless there is an alternative route for methionine biosynthesis. One possibility is a reverse trans-sulphuration pathway; e.g. synthesis of cystathionine from cysteine, 2-oxobutyrate plus ammonium. Such a pathway would not be present in *S. coelicolor* A3(2) (A21).

Homoserine dehydrogenase was reported to be present in *S. clavuligerus* (J71) and to be inhibited by 75% by threonine, 50% by homoserine and 20% by methionine. There was also some evidence of low-level enzyme repression by isoleucine and threonine and activation by lysine (Mendelovitz and Aharonowitz, 1982).

Nagasawa *et al.* (1984) could not find cystathionine β-lyase (cystathionase II), or O-acetylhomoserine sulphydrase (homocysteine synthase) in *S. phaeochromogenes* (A40). They were able to find cystathionine γ-synthase, the trans-sulphuration pathway enzyme which was capable of combining O-succinylhomoserine and cysteine or O-acetylhomoserine and cysteine. Therefore, how does the streptomycete synthesize homocysteine? There were two possibilities: the cystathionine γ-lyase was also capable, at one-seventh the efficiency of the α,γ-elimination reaction, of α,β-elimination of cystathionine producing pyruvate, homocysteine and ammonium; and there was an activity that could condense O-succinylhomoserine and sodium hydrosulphide to form homocysteine directly. It was not clear if the latter activity was a side reaction of cystathionine γ-synthetase as in *Salmonella* sp. The authors did show cystathionine γ-synthetase did not have an O-acetylhomoserine sulphydrylase activity. Nothing on the regulation of these enzymes was reported.

Young and Smith (1975) reported the isolation of methionine-overproducing mutants of *S. fradiae* (G68) using the analogue ethionine. This

implies that methionine metabolism is regulated and the system can be altered by mutation, but it need not mean there is gene regulation; i.e. feedback repression or attenuation (see section 4.4).

Vargha *et al.* (1986) reported that a mutant of *S. fradiae* (G68) appeared to have a specific requirement for methionine for sporulation. The mutant was apparently not a methionine auxotroph in the usual sense, but it could have been a bradytroph. The authors speculated that the mutation affected the last enzyme of methionine biosynthesis, homocysteine methyltransferase, as homocysteine could not replace methionine. They also suggested there was a particular requirement for methionine during sporulation, possibly as a methyl donor. The final enzyme of methionine biosynthesis, homocysteine methyltransferase (Fig. 7), was characterized in *S. olivaceus* (A1C) and shown to be vitamin B_{12}-dependent (Ohmori *et al.*, 1971). The *metH* gene, which encodes the methyltransferase, has been shown to be next to the *S. coelicolor* A3(2) (A21) *gylCABX* operon (section 2.2.4.1) (Redenbach *et al.*, 1996).

The gene *metF* – encoding the enzyme 5,10-methylenetetrahydrofolate reductase that is responsible for regeneration of 5-methyltetrahydrofolate, the methyl donor in the homocysteine methyltransferase reaction – has been characterized in *S. lividans* (A21) (Blanco *et al.*, 1998). Disruption of the gene generated a Met^- mutant. The enzyme is translated from a leaderless mRNA and there is a region upstream of the *metF* promoter that looks like a binding site for MetR, the methionine regulator of *Salmonella typhimurium*.

4.1.8.4. S-Adenosyl-Methionine. A role of methionine, other than in protein synthesis, is as a precursor of S-adenosyl-methionine (SAM) the major methyl group donor. Once the methyl group has been donated, S-adenosyl-homocysteine (SAHC) can be dealt with in a number of ways in streptomycetes. Shimizu *et al.* (1984) reported the presence of an SAHC hydrolase which hydrolysed the compound to adenosine and homocysteine in *S. hygroscopicus* (A32), *Streptoverticillium kentuchense* (F single member cluster) and representatives of the genera *Arthrobacter*, *Mycobacterium*, *Microellobospora*, *Micromonospora*, *Micropolyspora*, *Nocardia* and *Streptosporangium*. Presumably, homocysteine was recycled in the synthesis of methionine. SAHC hydrolase was previously thought to be present only in eukaryotes (Walker and Duerre, 1975).

The enzyme usually associated with SAHC catabolism in prokaryotes, SAHC nucleosidase, which generates adenine and S-ribosyl-homocysteine, has not been identified in streptomycetes so far. Another SAHC hydrolysing enzyme, SAHC deaminase, was identified in *S. flocculus* (A16) (Speedie *et al.*, 1988; Zulty and Speedie, 1989). The deamination of SAHC forms S-inosyl-homocysteine and is thought to play an important role in the synthesis of streptonigrin, because removal of SAHC removes a potent inhibitor of the essential methylase involved in the synthesis of this secondary metabolite.

The fate of S-inosyl-homocysteine is not clear. These authors also identified a low level of SAHC hydrolase in the cell extract.

4.2. Osmotic Stress Response in Streptomycetes

Streptomycetes are unusual in their resistance to osmotic stress. *S. coelicolor* A3(2) (A21) can grow, albeit poorly, on solid media containing 1.5 M NaCl (Wood, 1996). Two streptomycetes, *S. griseus* (A1B) and *S. californicus* (A9), were isolated from saline soil and could grow in media containing 1 M NaCl (Killham and Firestone, 1984a). The growth rate and growth yield of both strains were reduced by osmotic stress and NaCl reduced these growth parameters more effectively than did the same concentration of KCl. The ability of cells to resist high osmotic pressure comes from the accumulation of compatible solutes within the cells. In enteric bacteria, the compatible solutes are betaine, amino acids and trehalose (Booth and Higgins, 1990). The two streptomycetes accumulated amino acids proportionate to the size of the salt challenge, up to 0.75 M NaCl. Beyond this limit there was little or no increase in intracellular amino acid concentration. In *S. californicus* (A9) the amino acid pool increased 9.33-fold from no salt to 0.75 M NaCl. The composition of the amino acid pool also altered as it increased. At maximum stimulation, proline accounted for about 50% of the free amino acid pool in both streptomycetes. Proline is a neutral, rather flat molecule and so is an ideal compatible solute. Glutamine was the next most significant amino acid, and alanine the next. In cells not subject to osmotic challenge, glutamate and aspartate predominated in the free amino acid pool (Killham and Firestone, 1984a).

As proline was such an important osmoprotectant, the effect of addition of proline to salt stressed *S. griseus* (A1B) was examined (Killham and Firestone, 1984b). Exogenous proline increased the growth yield of the streptomycete when challenged with NaCl. In cells challenged with 0.75 M NaCl, 55% of the amino acid in the pool was imported. This rose to 71% in 1 M-NaCl challenged cells. Import, however, was tightly regulated as the relative proportion of proline in the intracellular pool was not altered by the presence of exogenous proline. This implied that import of proline decreased the amount of *de novo* proline synthesis. The decrease in requirement for proline biosynthesis accounted for the increase in growth yield.

The important osmoprotection role of some amino acids needs to be considered when thinking about the regulation of amino acid catabolism and anabolism. The mechanism of activation of amino acid biosynthesis by osmotic challenge has not been characterized in streptomycetes.

It has been proposed that trehalose might act as an osmoprotectant in streptomycetes, but it has not been demonstrated that trehalose synthesis was activated by changes in osmotic pressure (section 2.3.1).

4.3. Nucleotide Biosynthesis

4.3.1. Pyrimidines

Little has been reported on the biosynthesis of pyrimidines in streptomycetes. Uncharacterized auxotrophic mutations (*thyA1*, *uraA*, *uraB* and *uraC*) have been isolated in *S. coelicolor* A3(2) (A21) (Hopwood *et al.*, 1973). The isolation of auxotrophic mutants whose nutritional requirements can be satisfied by uracil and arginine (*uraD*) implied a common intermediate in these two biosynthetic pathways. Carbamoyl phosphate is one such common intermediate. Therefore, the pathway of pyrimidine biosynthesis by *S. coelicolor* A3(2) (A21) might be similar to that in other bacteria and *uraD* may be equivalent to one of the carbamoyl phosphate synthase (*car*) genes of *E. coli*.

4.3.2. Purines

Yamada *et al.* (1990) reported the presence of three of the enzymes of adenine biosynthesis, 5′-phosphoribosyl-5-aminoimidazole (AIR) carboxylase, 5′-phosphoribosyl-4-(*N*-succinocarboxamide)-5-aminoimidazole (SAICAR) synthetase and adenylosuccinate (SAMP) lyase, in *S. azureus* (A18) grown on minimal medium containing adenine. They also demonstrated that two adenine auxotrophs (Ade2 and Ade21) with altered spore pigmentation had lost AIR carboxylase and accumulated AIR. A further adenine auxotroph (ATH) that also needed thiamine was isolated and shown to have lost 5′-phosphoribosyl-5-amino-4-imidazole-carboxamide (AICAR) formyltransferase activity (Ogata and Yamada, 1990). The mutant accumulated AICAR on minimal medium (Ogata *et al.*, 1991). This implied that the pathway of purine biosynthesis was the same as in other bacteria.

Yamada *et al.* (1990) did not report any regulatory aspects of purine anabolism, but the adenine auxotrophs had about the same level of SAICAR synthetase and SAMP lyase activities as did the wild type, implying a lack of feedback gene repression. Strain ATH accumulated AICAR and Ade2 and Ade21 accumulated AIR. In medium containing excess adenine, all the mutants accumulated AICAR, which was a surprise as it implied that AICAR was synthesized in Ade2 and Ade21 in the absence of AIR carboxylase. Dephosphoribosylated AICAR reduced the accumulation of AIR in Ade2 and Ade21 and was thought to reduce AICAR accumulation in ATH and all mutants on adenine media (Ogata *et al.*, 1991). This implies that there was some kind of feedback regulation of this part of the purine biosynthesis pathway.

There was a salvage synthesis pathway for purines that allowed the synthesis of inosine monophosphate (IMP), and hence adenosine and guanosine

monophosphate, from hypoxanthine via hypoxanthine phosphoribosyl-transferase (HPRT) in *S. cyanogenus*. The pathway was active in nitrogen-replete conditions when the hypoxanthine oxidation pathway was repressed (Watanabe *et al.*, 1976b) (section 3.4.2).

4.4. Nitrogen Metabolite Biosynthesis: General Conclusions

The most unusual characteristic of amino acid biosynthesis in streptomycetes is the rarity of feedback gene repression by the product of the pathway. Table 16 and Fig. 8 summarize the data on feedback repression of amino acid biosynthesis. It should be noted that in a number of cases, *stimulation* of gene expression rather that repression was seen when the cognate amino acid was added to the culture medium.

The DAHP synthase (*aroH*) and anthranilate synthase (*trpEG*) of the aromatic amino acids, arginine biosynthesis and the branched-chain amino acids, appear to be regulated by feedback repression, and in some cases mechanisms have been found. Significant levels of repression (greater than five-fold) have been reported, although often with addition of very high levels of amino acid. Note that addition of an amino acid outside the cell does not necessarily result in the same concentration of the amino acid inside the cell. The transport system may be gated (section 3.2.2.2 – arginine transport is very poor at pH 7.0) and catabolism of the amino acid may be induced. A more certain way of examining repression levels is to use bradytrophs. These auxotrophs contain mutations that reduce enzyme activity rather than abolish it; thus the cell can grow in the absence of the anabolite, albeit more slowly. Such anabolite-limited growth will mean that there is no amino acid available to repress the biosynthetic genes and so full derepression can be expected. Bradytroph studies clearly showed the late *trp* genes, *trpD*, *trpC* and *trpBA*, were not subject to feedback control (Hu *et al.*, 1999) but that the early *trp* gene *trpEG* was subject to feedback control (Lin *et al.*, 1998).

There is very strong circumstantial evidence (section 4.1.3.2) that a regulator, ArgR, has been identified that is responsible for the repression of amino acid biosynthesis and activation of catabolism in streptomycetes. There is very slight circumstantial evidence that the *metF* gene may have a MetR-like binding site (section 4.1.8.3).

Potential attenuation sites have been identified for *trpEG* (section 4.1.1.4), and *leuA* and *ilvBNC* (section 4.1.4.3) and an attenuation peptide for rhodanese (section 4.1.8.1). There is no positive evidence that these potential sites for attenuation actually control gene expression, and in the *leuA* and *ilvBNC* there is overwhelming evidence that they do not (Craster *et al.*, 1999). However, there is also clear evidence that these branched-chain amino acid genes and all the others tested, which do not have potential attenuation sites,

Table 16 Levels of repression of amino acid biosynthesis in streptomycetes.

Enzyme	Repressor	Repression level	Strain	Reference
Arginine				
N-Acetylglutamyl phosphate reductase	Arginine	2	*S. clavuligerus* (J71)	Ludovice *et al.*, 1992
Ornithine carbamoyltransferase	Arginine	2.3	*S. clavuligerus* (J71)	Padilla *et al.*, 1991
	Arginine	1.5	*S. coelicolor* A3(2) (A21)	Padilla *et al.*, 1991
	Arginine	47.1[a]	*S. coelicolor* A3(2) (A21)	Flett *et al.*, 1987
	Arginine	2.4	*S. griseus* (A15)	Padilla *et al.*, 1991
	Arginine	3.4	*S. lividans* (A21)	Padilla *et al.*, 1991
Argininosuccinate synthase	Arginine	37.5[a]	*S. coelicolor* A3(2) (A21)	Flett *et al.*, 1987
Argininosuccinase	Arginine	7.3[a]	*S. coelicolor* A3(2) (A21)	Flett *et al.*, 1987
Aromatic amino acid				
DAHP synthase	Tryptophan	11	*S. antibioticus* (A31)	Murphy and Katz, 1980
	None		*S. aureofaciens* (A14)	Gorisch and Lingens, 1971
	Anthranilate	2	*S. venezuelae* (A6)	Lowe and Westlake, 1971
Chorismate mutase	None		*S. aureofaciens* (A14)	Gorisch and Lingens, 1973
	None		*S. venezuelae* (A6)	Lowe and Westlake, 1972
				Gorisch and Lingens, 1972
Anthranilate synthase	Tryptophan	9.5	*S. venezuelae* (A6)	Lin *et al.*, 1998
	Tryptophan	12	*S. parvulus* (A12)	Katz *et al.*, 1984
Anthranilate phosphoribosyl transferase	None		*S. coelicolor* A3(2) (A21)	Hu *et al.*, 1999

Table 16 cont.

Enzyme	Repressor	Repression level	Strain	Reference
Indoleglycerol phosphate synthetase	None		*S. coelicolor* A3(2) (A21)	Hu *et al.*, 1999
Tryptophan synthase	None		*S. coelicolor* A3(2) (A21)	Hu *et al.*, 1999
Aspartate-family amino acids				
Aspartokinase	Isoleucine	3.4	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
	Methionine	2.2	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
	Homoserine	1.1	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
	Diaminopimelate	1.1	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
	Threonine	None	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
	Lysine	1.2	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
	Methionine	3	*S. fradiae* (G68)	Vargha, 1997
Dihydropicolinate synthetase	Diaminopimelate	1.3	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
	Isoleucine	1.2	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
	Lysine	None	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
Homoserine dehydrogenase	Isoleucine	1.8	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
	Threonine	1.2	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
	Lysine	1.2	*S. clavuligerus* (J71)	Mendelovitz and Aharonowitz, 1982
Branched-chain amino acids				
Threonine dehydratase	Isoleucine	1.6	*S. fradiae* (G68)	Vancura *et al.*, 1989a
	Valine	2.5	*S. fradiae* (G68)	Vancura *et al.*, 1989a
	Leucine	1.3	*S. fradiae* (G68)	Vancura *et al.*, 1989a
	Threonine	1.3	*S. fradiae* (G68)	Vancura *et al.*, 1989a

Table 16 cont.

Enzyme	Repressor	Repression level	Strain	Reference
Acetohydroxy acid synthase (*ilvBN*)	Isoleucine	2	*S. fradiae* (G68)	Vancura *et al.*, 1989a
	Threonine	1.3	*S. fradiae* (G68)	Vancura *et al.*, 1989a
	Leucine	1.2	*S. fradiae* (G68)	Vancura *et al.*, 1989a
	Valine	↑1.2	*S. fradiae* (G68)	Vancura *et al.*, 1989a
	Isoleucine	↑1.2 2–3	*S. coelicolor* A3(2) (A21)	Potter and Baumberg, 1996
	IVL[b]	5–8	*S. coelicolor* A3(2) (A21)	Potter and Baumberg, 1996
	IVL[b]	16–23	*S. coelicolor* A3(2) (A21)	Craster *et al.*, 1999
Dihydroxy acid dehydrase (*ilvD*)	IVL[b]	17–30	*S. coelicolor* A3(2) (A21)	Craster *et al.*, 1999
Isopropylmalate synthase (*leuA*)	Isoleucine	2–3	*S. coelicolor* A3(2) (A21)	Potter and Baumberg, 1996
	IVL[b]	5–8	*S. coelicolor* A3(2) (A21)	Potter and Baumberg, 1996
Isopropyl malate isomerase (*leuCD*)	IVL[b]	11–93.5	*S. coelicolor* A3(2) (A21)	Craster *et al.*, 1999
β-isopropyl malate dehydrogenase (*leuB*)	IVL[b]	32–46	*S. coelicolor* A3(2) (A21)	Craster *et al.*, 1999
Glutamate and glutamine				
Glutamate synthetase	Glutamine	None	*S. coelicolor* A3(2) (A21)	Fisher and Wray, 1989
	Glutamine	9.8	*S. clavuligerus* (J71)	Bascaran *et al.*, 1989a
GOGAT	Aspartate + glutamine	3.8	*S. coelicolor* A3(2) (A21)	Fisher, 1989
	Glutamine	1.3	*S. clavuligerus* (J71)	Brana *et al.*, 1986a
Glutamate dehydrogenase	Aspartate + glutamine	2.8	*S. coelicolor* A3(2) (A21)	Fisher and Wray, 1989

Table 16 *cont.*

Enzyme	Repressor	Repression level	Strain	Reference
Histidine				
Phosphoribosyl-ATP -pyrophosphatase	Histidine	2.9	*S. coelicolor* A3(2) (A21)	Russi *et al.*, 1973; Derkos-Sojak *et al.*, 1985
Histidinol dehydrogenase	Histidine	2.5–4.9		
Imidazol-glycerol dehydratase	Histidine	2.7–4.9		
Histidinol phosphate phosphatase	Histidine	5.0		
Proline				
γ-glutamyl kinase	Proline	2	*S. coelicolor* A3(2) (A21)	
Glutamic-γ-semialdehyde dehydrogenase	Proline	2	*S. coelicolor* A3(2) (A21)	Hood *et al.*, 1992
Δ^1-pyrroline-5-carboxylic acid reductase	Proline	2	*S. coelicolor* A3(2) (A21)	
Sulphur amino acids				
Cystathionine γ-lyase	sulphate	3	*S. lactamdurans* (A7)	Kern and Inamine, 1981

[a]Comparison of enzyme levels in wild type and an Arg mutant; [b] IVL = isoleucine + valine + leucine; ↑ = induced.

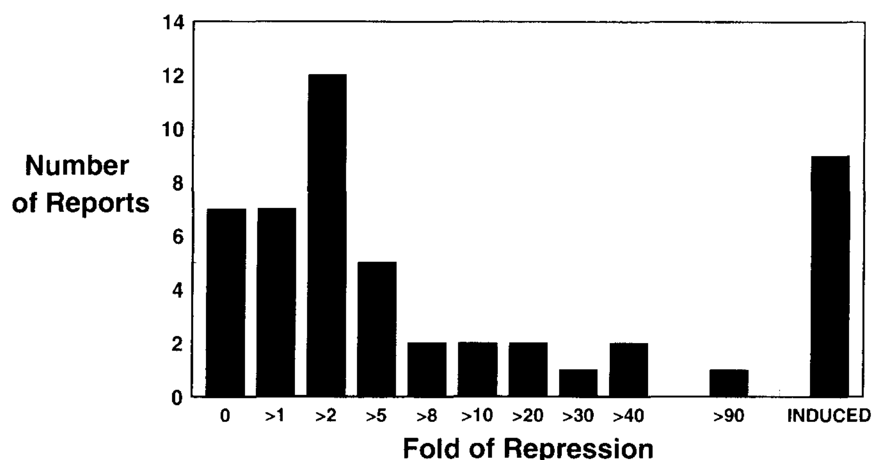


Figure 8 Repression of amino acid biosynthesis in streptomycetes (see Table 16 for data and references). Best repressor chosen if more than one.

were feedback-regulated by the cognate amino acids but no clue as to mechanism was found.

It is very difficult to decide what significance to attach to two-fold to five-fold repression and activation levels. One is tempted to dismiss it. However, in histidine biosynthesis in *S. coelicolor* A3(2) two mutants have been isolated that abolish low-level repression. The loss of repression caused by *hisC119–O*^{c} was interpreted as an operon promoter change as only those genes present in the same genetic locus were affected. In the second mutant, derepression occurred in genes at two chromosomal locations and the level of derepression equalled the specific amount of repression by histidine (two-fold or five-fold). The lesion was mapped to a different location than the previously mapped *his* genes (section 4.1.2). The ability to isolate such mutations and their phenotype implies that the low level of repression was due to specific genetic mechanisms that could be mutated.

There was evidence that the regulation of the late genes of tryptophan biosynthesis was not via feedback repression but as a response to the growth rate and growth phase of the culture (Hu *et al.*, 1999) (section 4.1.1.4). Maximal expression of the *trpD*, *trpC* and *trpBA* genes occurred in the early exponential phase. Therefore, tryptophan biosynthesis was subject to a global regulatory system, whereby maximal production was correlated with maximum need; i.e. when the cell was growing at its fastest. Proline biosynthesis is not subject to similar regulation in *S. coelicolor* A3(2) (A21) (D. Hood, personal communication), but other amino acid systems have not been examined. Other examples of growth phase regulation in streptomycetes involve the

activation of genes as the culture reaches the stationary phase. Examples include amino acid transport systems (section 3.2.2), alanine dehydrogenase of *S. hygroscopicus* (A32) (section 3.3.6), proteases of *Streptomyces* sp. C15, *S. lactamdurans* (A7) and *S. peucetius* (?) (section 3.1.1), and secondary metabolism (Strauch *et al.*, 1991).

The general observation that expression of streptomycete amino acid biosynthesis pathways is rarely feedback-regulated, or regulated to a small extent, explains the observation made by many researchers that it is often very difficult to assay streptomycetes' primary metabolism biosynthetic enzymes. Particular examples are the arginine system (Padilla *et al.*, 1991), *p*-aminobenzoate synthase (section 4.1.1.5) and proline biosynthesis (D.D.S. Smith, personal communication). This is often reflected in the large (1000-fold) purification factors necessary to obtain pure streptomycete amino acid biosynthesis enzymes, e.g. DAHP synthase (Stuart and Hunter, 1993). The implication is that many amino acid biosynthetic enzymes are constitutive but expressed at a low level.

The lack of regulation of amino acid biosynthesis at the genetic level might suggest that isolation of mutants in amino acid overproduction would be difficult. In other bacteria (e.g. enteric bacteria) loss of the amino acid gene repressor or deletion of the attenuation system will lead to overproduction. Amino acid overproduction mutants have been isolated from streptomycetes (Godfrey, 1973; Young and Smith, 1975; Vezina, 1976; Derkos-Sojak *et al.*, 1977; Vezina *et al.*, 1979; Aharonowitz *et al.*, 1984; Pospisil *et al.*, 1984, 1998; Vancura *et al.*, 1989a) by the isolation of auxotroph revertants and amino acid analogue-resistant mutants. Amino acid overproduction mutants selected as analogue-resistant can arise either (1) by altering a regulation system such that amino acid biosynthesis is no longer feedback-regulated by the analogue and the amino acid, or (2) by causing overproduction by some other means, such as loss of enzyme regulation, and thus swamping out the analogue. However, it is worth noting that amino acid overproduction can arise in cells with constitutively produced amino acid biosynthesis pathways by the loss of feedback inhibition of enzymes and the loss of amino acid catabolism genes. As was seen in section 3.3, catabolism genes are often constitutive in streptomycetes (Fig. 5), and so loss of the pathway or loss of an activator of the regulated pathways would lead to amino acid overproduction mutants.

In all the reports of gene sequences and enzyme characterization of primary metabolism in streptomycetes, an interesting common feature has been noted. In enteric bacteria, some of the enzymes in the shikimate, tryptophan, tyrosine, phenylalanine and histidine biosynthesis pathways, and proline catabolism pathways, are produced from fused genes. All the equivalent enzymes from streptomycetes are carried on separate peptides. The single exception is *trpEG*. Gene fusion is very common in genes involved in secondary metabolism.

Another unusual feature of streptomycetes amino acid biosynthetic genes is the occurrence of small open reading frames scattered amongst them. Examples occur in the *S. coelicolor* A3(2) histidine (Limauro *et al.*, 1990), tryptophan and proline (Hood *et al.*, 1992) genes, and the *Saccharopolyspora erythraea* cysteine genes (Donadio *et al.*, 1990). It is tempting to speculate that these may have some role in regulation. However, there is as yet no evidence for such a role.

In summary, streptomycetes' nitrogen anabolism appears to use many of the pathways present in other bacteria. The exceptions are the arogenate pathway of tyrosine biosynthesis and the trans-sulphuration pathway of methionine and cysteine interconversion. The regulation of these pathways is often different from that seen in other bacteria. Amino acid biosynthesis often appears to be constitutive, but at a low level when compared with other bacteria. Where regulation does occur, it appears to be novel and worthy of investigation. There has been a tendency in the literature to assume that what is true for enteric bacteria is also true for streptomycetes. Hopefully this section demonstrates that that assumption is often wrong; and that if we wish to exploit streptomycete primary metabolism for improvements in the yield and diversity of secondary metabolites, we must obtain a more accurate understanding of its regulation.

5. STREPTOMYCETE PRIMARY METABOLISM: SOME CONCLUSIONS

As discussed in section 1.2, streptomycetes appear to be facultative oligotrophs. The control of primary metabolism may be taken as a reflection of the ecological niche of these bacteria. Streptomycetes normally live in soil, which is a nutrient-limited and competitive environment. Soil is dependent on plant productivity for input of nutrients, and plants tend to be carbon-rich owing to the ready availability of CO_2 for fixing, but nitrogen-limited. The saprophytic streptomycetes are, therefore, going to be nitrogen-limited rather than carbon-limited. Examination of the control of carbohydrate catabolism reveals a plethora of inducible carbohydrate catabolic systems, including extracellular enzymes for degradation of insoluble polymers, some to gain access to utilizable compounds, some to release such compounds, transport systems for these soluble products of digestion, and intracellular catabolic pathways. The wide range of catabolic pathways observed and studied reflects both the wide-range of utilizable carbohydrate substrates available in soil and the biotechnological interest in streptomycetes as suppliers of enzymes to industry. The multitude of catabolic systems in the streptomycetes, and the carbohydrate resources available in soil, implies a strong need for coordinate control of carbohydrate catabolism. This appears to be reflected in the carbon catabolite repression

system in streptomycetes. This system, while not yet well understood, and different from what is found in other bacteria, is clearly widespread and wide-ranging in its control.

An interesting observation was made when examining carbohydrate transport. Constitute permeases had low affinity but high capacity whilst inducible permeases had high affinity (Tables 3 and 4). The low-affinity permeases probably reflect again the relatively carbohydrate-replete conditions. If carbohydrate polymers are being degraded *in situ*, we might expect relatively high local concentrations of monosaccharides and disaccharides. Amino acid transport, on the other hand, appears to utilize mainly high-affinity transport systems (Table 11), which can be rationalized by the relatively amino acid-deficient environment of soil.

When we turn to amino acid and nucleotide catabolism, it appears that half of the pathways are inducible and half are expressed at low levels and constitutive. This, and the observation that streptomycetes do not often exploit feedback regulation of amino acid metabolism, may be a reflection of their ecological niche (i.e. the soil environment), which will be relatively poor in nitrogen catabolites. When we compare soil to an alimentary canal, the latter will be replete in amino acids; thus enteric bacteria might be expected to have more coordinating control mechanisms than soil bacteria. Yet because the enteric bacteria and other copiotrophs have been studied so well, we tend to think that the absence of such systems is a problem for the streptomycetes, rather than appreciate that the physiology of enteric bacteria and streptomycetes, is bound to be different. In other words, the streptomycetes can pick and choose their carbon and energy sources because they are available in excess, whilst the nitrogen sources are limited and they must grab what they can. If amino acids are often not available, streptomycetes will have to synthesize them and so the amino acid biosynthesis shutdown mechanisms did not evolve or have been lost.

A similar absence of repression of amino acid biosynthesis has been seen in *Caulobacter crescentus* (Ross and Winkler, 1988). This Gram-negative bacterium also lives in a low-nutrient environment; i.e. ponds and rivers. There was evidence that cysteine biosynthesis was tightly regulated (Bellofatto *et al.*, 1984), which might not be too surprising as this amino acid is involved in carbon, sulphur and nitrogen metabolism. We might expect the regulation of biosyntheses of sulphur-containing amino acids in streptomycetes to be similarly tightly regulated (section 4.1.8).

It is clear that many amino acid biosynthetic pathways are constitutive and amino acid catabolism is constitutive. This raises the interesting question: how do streptomycetes avoid futile cycling of amino acids? Do the catabolism and amino acid consumption pathways (i.e. protein synthesis, etc.) have different affinities for their substrate? The streptomycetes, like all other cells, contain pools of free amino acids and the lack of catabolism needs to be

explained. We have shown that loss of proline catabolism in a cell that constitutively produces proline leads to activation of a secondary metabolic pathway that utilizes proline (Hood *et al.*, 1992). This reveals another important aspect of streptomycete physiology, the presence of tightly regulated secondary metabolism. If nitrogen catabolism and biosynthesis are less tightly regulated than in other bacteria, the old idea of secondary metabolism as a form of overflow metabolism may be due for revival (section 1.6). The change in the concept is that primary metabolism is relatively poorly controlled whereas secondary metabolism is tightly controlled. A biological role for secondary metabolism may be to deal with unbalanced production of primary metabolites when streptomycetes are supplied with unusual 'feast' conditions. The normal status for streptomycetes in soil will be for famine of nitrogenous compounds and feast of carbohydrates. If that situation is reversed, it appears that streptomycetes do not have the regulatory capacity to modify their primary metabolism. If, however, secondary metabolic pathways can be induced that can deal with the specific anabolite glut, it can be seen that advantage can be gained by the cell, independent of the chemical nature of the final secondary metabolite (section 1.6.4).

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Adaptation of Oral Streptococci to Low pH

Robert G. Quivey Jr, Wendi L. Kuhnert and Kristina Hahn

Department of Microbiology and Immunology and the Center for Oral Biology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642 USA

ABSTRACT

The strategies employed by oral streptococci to resist the inimical influences of acidification reflect the diverse and dynamic niches of the human mouth. All of the oral streptococci are capable of rapid degradation of sugar to acidic end-products. As a result, the pH value of their immediate environment can plummet to levels where glycolysis and growth cease. At this point, the approaches for survival in acid separate the organisms. *Streptococcus mutans*, for example, relies on its F-ATPase, to protect itself from acidification by pumping protons out of the cells. *S. salivarius* responds by degrading urea to ammonia and *S. sanguis* produces ammonia by arginolysis. The mechanisms by which these organisms regulate their particular escape route are now being explored experimentally. The picture that emerges is that the acid-adaptive regulatory mechanisms of the oral streptococci differ markedly from those employed by Gram-negative bacteria. What remains to be elucidated are the breadth of the acid-response systems in these organisms and how they permit the microbes to sustain themselves in the face of low pH and the bacterial competition present in their respective niches. In this article, we summarize reports concerning the means by which oral streptococci either utilize acidification to subdue their competitors or protect themselves until pH values return to a more favorable level.

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1. INTRODUCTION

The oral streptococci are a diverse group of organisms that occupy dynamic ecological niches in the human mouth. Some members of the group, the mutans streptococci, participate in the development of one of man's most common afflictions, dental disease (Loesche, 1986). Other members of the oral streptococci, such as *S. sanguis*, are considered to be commensal organisms when they grow on the surfaces of teeth and pathogenic when they make their way to cardiac tissue (Herzberg *et al.*, 1997). As a collection, the oral streptococci inhabit the surfaces of teeth, colonize the epithelium of the cheeks, and persist in the folds of the tongue, all of which present differing stresses for the organisms. The present discussion will provide a view of the advances made in our understanding of how the oral streptococci respond to one of their most significant challenges, survival in the face of fluctuating pH values, brought about largely by their own metabolic efforts.

The results of physiological studies, biochemical investigations, animal model experiments, and the implementation of molecular approaches have provided insights into the mechanisms by which oral streptococci adapt to pH changes in the mouth. Now, as genomic information becomes available for several oral organisms and biofilm technology becomes more widely applied, proteomics will serve to broaden our understanding of how the streptococci respond via global genetic regulation to pH changes and the presence of other bacteria in their environment.

Early emphasis in the field was on simple identification of the organisms participating in the development of oral disease and an understanding of the complex relationships that apparently exist in the oral cavity. More recently, interest has grown to include questions regarding the molecular nature of the mechanisms conveying acid-resistance in the oral streptococci. Eventually, the goal will be to apply our knowledge of molecular mechanisms to the interactions of oral bacteria with one another and with the human host tissues on which they reside.

1.1. Ecology of Oral Acid Production

Initially, in the mouth, tooth surfaces and soft tissues are bathed in saliva, which contains acidic glycoproteins (mucins). Mucins adhere to the tooth surface along with other salivary constituents to form a thin film, referred to as the acquired enamel pellicle (AEP) (Tabak, 1995). Strains of streptococci selectively adhere via specific hydrophobic or lectin-like interactions (Jenkinson and Lamont, 1997). Subsequently, co-aggregation involving organisms such as *Actinomyces viscosus* or *A. naeslundii* with *Streptococcus sanguis* can occur (Cisar, 1982). As organisms begin to accumulate, *Streptococcus mutans* colonizes the growing microbial consortium and becomes irreversibly bound in the presence of sucrose. Sucrose, provided by the human diet, is not only metabolized by *S. mutans*, but is also used to produce extracellular, adherent glucans and fructans (Gibbons and van Houte, 1980; Hamada and Slade, 1980; Staat *et al.*, 1980). The glucan polymers, synthesized by glucosyltransferases (GTFs) and fructosyltransferases (FTFs), contribute to the cariogenicity of *S. mutans* (Gibbons and van Houte, 1975; Fukushima *et al.*, 1981; Fukui *et al.*, 1982; Kuramitsu and Wondrack, 1983; Munro *et al.*, 1991; Yamashita *et al.*, 1993a)

As each of the above components – salivary constituents, food particles and bacteria – accumulate, a new ecological niche, dental plaque, is formed (Nikiforuk, 1985). Studies have shown that while dental plaque consists of up to 300 species of bacteria, a relatively small number are odontopathogens (Moore *et al.*, 1982a, b, 1983; Socransky *et al.*, 1982). The oral lactic acid bacteria, *Streptococcus mutans*, along with *Lactobacillus casei*, have been implicated in the production of caries and are found associated with carious lesions (Loesche, 1982; Emilson and Krasse, 1985).

1.2. Acid-Adaptive Strategies

Consideration of how the streptococci survive in the oral cavity requires a few preliminary comments about the nature of their environment and why acid-adaptive strategies are necessary for persistence of the organisms. The major, degradative products of sugar metabolism by oral streptococci are organic acids, particularly lactic acid. The accumulation of lactate results in a rapid and significant decrease in localized pH of the plaque environment, with values reported as low as pH 4.0,which is well below the value at which demineralization of tooth enamel begins to occur (Jensen *et al.*, 1982). Supporting studies, conducted in animal models, have shown the onset of dental caries occurs after the introduction of sucrose into the diet (Keyes, 1968; Tanzer, 1981; Fitzgerald *et al.*, 1994). In humans, a shift in the microbial populations toward increased numbers of the pathogenic *S. mutans* and decreased numbers of the non-pathogenic *S. sanguis* can be seen when high-sucrose diets are

initiated (Dennis *et al.*, 1975; Staat *et al.*, 1975; Skinner and Woods, 1984; Minah *et al.*, 1985). *In vitro* studies, with mixed bacterial cultures, have demonstrated that the proportion of *S. mutans* increases while that of *S. sanguis* decreases at low pH (Bowden and Hamilton, 1987; Bradshaw *et al.*, 1996). Nevertheless, *S. sanguis*, and other organisms, which are not as acidogenic nor as aciduric as *S. mutans*, continue to survive in the mouth. Thus, it is clear that streptococcal survival strategies have evolved to either take advantage of acidurance or to endure acidification until pH values in the mouth rise.

2. ROLE OF THE F-ATPase IN ACID ADAPTATION

2.1. Physiology of Oral Streptococcal ATPase

A great deal of our present understanding of the importance of acid-adaptation to the oral streptococci has come from studies of the properties of the membrane-bound, proton-translocating ATPase (F-ATPase). Early experiments, conducted with *Enterococcus hirae* (formerly *Streptococcus faecalis*), showed that levels of the F-ATPase were up-regulated during growth at pH values around 5.0 (Kobayashi *et al.*, 1982, 1984, 1986; Abrams and Jensen, 1984; Kobayashi, 1985). Collectively, the work indicated that the actual amount of F-ATPase present in cell membranes somehow increased during acidic growth. Moreover, it appeared that the primary role of the enzyme was to provide a mechanism for pH homeostasis for the cells. Unlike the enteric bacteria, which have a relatively stable internal pH (pH_i), the streptococci allow the pH_i to float relative to the external environment, relying on the F-ATPase to pump protons out of the cell. For example, the pH_i of *S. mutans* is 0.5–1 pH units more alkaline than the external environment (Dashper and Reynolds, 1992).

Attempts to understand the nature of acidurance in oral streptococci have led to the observation that there are intrinsic differences in the abilities of *S. mutans*, *S. salivarius*, and *S. sanguis* to continue glycolysis and growth under acidic conditions. A hierarchy has been established for the minimum pH values at which growth and glycolysis can occur: 4.8 and 4.4, respectively for *S. mutans*; 4.9 and 4.3, respectively for *S. salivarius*; and, 5.2 for both growth and glycolysis in *S. sanguis* (Harper and Loesche, 1983, 1984; Bender *et al.*, 1985). The data show that the streptococci are capable of forming ATP (from glycolysis) at levels well below that at which the cells are capable of growing, a point that will become more significant later in this discussion. Treatment of oral streptococcal strains with the protonophore, gramicidin, resulted in higher minimum pH values for glycolysis, establishing that the streptococcal membrane provides an important barrier function for protection of not only glycolytic enzymes but other cellular components (Bender *et al.*, 1985).

Subsequent work showed that the differences in the abilities of *S. mutans*, *S. salivarius* and *S. sanguis* to carry out glycolysis and survive acid challenge actually were more dependent on their abilities to remove protons from the cytoplasm by the action of the F-ATPase. Experiments with the ATPase inhibitor, dicyclohexylcarbodiimide (DCCD), showed that reducing the enzyme's activity also resulted in an increase in membrane permeability to protons (Bender *et al.*, 1986). Thus, a dynamic effect was described wherein protons flow into the streptococci during growth at low pH and are removed via ATP-dependent proton pumping. Clearly, then, the ability of the cells to form ATP at low pH values becomes critical for survival until pH rises to levels at which growth can be resumed.

In work performed with isolated membranes, pH optima were established for *S. sanguis*, *S. salivarius*, *S. mutans*, and *L. casei*, of approximately 7.5, 7.0, 6.0, and 5.0, respectively (Bender *et al.*, 1986). In this way, it was shown that the acid tolerances of oral streptococci revolve around the relative abilities of an organism's F-ATPase to function at increasingly lower pH values, and that intracellular pH homeostasis is the major function of the enzyme. The central role of ATPase is also seen in the enteric bacteria, where it has been shown that the acid-tolerance response (ATR) does not occur in cells that are defective in the F-ATPase (Foster and Hall, 1991). The likelihood exists, then, that ATPase function is a key component of acid tolerance in bacteria in general.

The magnitude of the differences in ATPase pH optima between oral bacteria was made more manifest when it was discovered that these organisms contain significant differences in the amounts of the enzymes that are produced. For example, the relatively non-aciduric organism *Actinomyces viscosus* contains approximately 50-fold less ATPase protein than the highly aciduric *Lactobacillus casei* (Bender and Marquis, 1987). *S. mutans* and *S. sanguis* fall between *A. viscosus* and *L. casei*, with *S. mutans* producing somewhat greater amounts of the enzyme than *S. sanguis* (Sutton and Marquis, 1987). Thus, as the pH optimum of the *S. mutans* ATPase provides a competitive advantage during growing in dental plaque, the relatively greater abundance of the enzyme contributes an additional ecological benefit to the organism.

Initial biochemical studies on the nature of the F-ATPase from oral streptococci indicated that the enzymes contain the same number and mass of subunits as the well-described enzyme from *E. coli* (Sutton and Marquis, 1987). The streptococcal enzymes were inhibited by fluoride and DCCD, but not by ouabain or vanadate, establishing that the activities were indeed of the F-type (Sutton and Marquis, 1987). The enzyme from *S. mutans* proved to be more sensitive to fluoride-mediated inhibition than that from *L. casei*, 3 mM versus 50 mM, respectively for 50% inhibition of activity (Sutton *et al.*, 1987), though the significance of the observation is not yet entirely understood. At low levels of fluoride the inhibition was reversible; and high levels of ATP or

magnesium were effective at relieving the inhibition, suggesting that the action of fluoride might be at the active site of the enzyme. Later work showed that the inhibition of the ATPase by fluoride was dependent on the presence of aluminum (Al^{3+}), which apparently formed fluoride complexes that spatially resemble ATP (Sturr and Marquis, 1990). Fluoride–metal complexes also inhibit the F-ATPase from *E. coli,* suggesting the strong likelihood of conservation at the level of catalytic sites, at least (Weber and Senior, 1997). Results from studies with isolated F_1F_0 complexes showed that the enzymes from *S. mutans, S. sanguis,* and *L. casei* exhibited similar sensitivities to aluminum-fluoride (AlF_4) complexes. The pH optima for the isolated enzymes were very similar to that found for the activities expressed in whole cells, reinforcing the concept that the differences in the enzyme activities were in fact due to the proteins themselves and not to factors found elsewhere in the cells (Sturr and Marquis, 1992). Interestingly, however, the pH optima for isolated ATPases narrowed considerably for the F_1 complex alone, suggesting that the membrane subunits, or their interaction with the membrane itself, might play a significant role in modulating the aciduricity of the enzyme, and hence the organism (Sturr and Marquis, 1992).

The relative acidurance of oral streptococcal species is clearly based on the amount and pH optima of the ATPase, which provides an inherent level of acid tolerance. The facilitated movement of protons, via the F-ATPase, results in an internal pH which is more basic than that of the plaque environment. Thus, the enzyme acts to protect relatively acid-sensitive glycolytic enzymes – explaining, in part, the mechanism by which *S. mutans* is able to survive within the oral cavity and promote dental caries (Casiano-Colon and Marquis, 1988; Dashper and Reynolds, 1992). However, the cell's reliance on the enzyme apparently does not reside entirely at the level of biochemistry. That is, it has been shown that *S. mutans* and *S. sanguis* are also able to increase the amount of ATPase that they produce approximately 2- to 4-fold, in response to growth at low pH values (Belli and Marquis, 1991; Hamilton and Buckley, 1991; Kuhnert and Quivey, 1999). Thus, the situation, for enzymatic activity, is similar to that described for the ATPase from *E. hirae* (Kobayashi *et al.*, 1984). The apparent up-regulation of ATPase production indicates that the organisms have the capacity to induce a response(s) to growth at low pH, possibly including mechanisms similar to those described for the enteric and other bacteria (Foster, 1995; Hall *et al.*, 1995; Rowbury *et al.*, 1998). Along with the description of ATPase up-regulation, Marquis *et al.* also noted that the adaptation extended to glycolytic capacity, in that the minimum pH at which glycolysis occurs was lowered by approximately 0.5 pH units in cells growing at pH values around 5.0 (Belli and Marquis, 1991). The mechanism of the change in acid-sensitivity of glycolysis is unclear. However, it is tempting to speculate that chaperonin interaction with glycolytic enzymes might provide stability to the enzymes in the presence of an acid challenge (see

below). It may also be that additional, adaptive, change occurs in the membranes, resulting in additional protection of the glycolytic enzymes from acidification. The relatively small amount of data available on this point suggest that the organism is capable of responding to thermal stress by changing its use of exogenously supplied fatty acids, with the result that *S. mutans* becomes more acid-resistant in the presence of lauric (C12) or palmitic (C16) acids (Ma and Marquis, 1997).

2.2. Organization of the Streptococcal ATPase Operons

A vast amount of literature has been generated with regard to the structure, function and regulation of the F-ATPase in other bacteria and the interested reader is urged to examine recent reviews on the topic (Fillingame, 1992; Weber and Senior, 1997; Fillingame *et al.*, 1998). The volume of information available provides a powerful backdrop against which to consider the role of the ATPase in the relative aciduricity of the oral streptococci.

Availability of the *B. megaterium* (Brusilow *et al.*, 1989) and *E. coli* F-ATPase (Walker *et al.*, 1984) operon nucleotide sequences greatly assisted the cloning of the homologous operons from *S. mutans* and *S. sanguis* (Smith *et al.*, 1996; Kuhnert and Quivey, 1999). The deduced amino acid sequences for the eight structural genes of the streptococcal ATPase operons showed that the enzymes are homologous to the well-characterized subunits from the *E. coli* ATPase, as well as those of other bacteria (Smith *et al.*, 1996). In general, the subunits in the F_1 (cytoplasmic domain) showed a higher level of homology than the F_0 (membrane domain). The genetic organization of the streptococcal operons was identical to that shown for the *E. hirae* operon (Shibata *et al.*, 1992; Smith *et al.*, 1996; Kuhnert and Quivey, 1999). Partial operon sequences are available from *S. oralis* and *S. pneumoniae* (Fenoll *et al.*, 1994), and the entire operon from *S. pyogenes* is available from the genomic database (Roe *et al.*, 1999). To date, all streptococci appear to possess an F_0 gene order consisting of *atpEBF*, as compared with *atpBEF* seen in most other bacteria. The significance of the altered gene order for the membrane-bound subunits is unclear, though results from *E. coli* indicate that rearrangement of the subunits does not appear to influence assembly and function of the enzyme (Weber and Senior, 1997). Interestingly, neither the *S. mutans* nor the *S. sanguis* operon contained an *atpI* gene-equivalent upstream of the structural genes. Instead, for example, the *S. mutans* operon has an intergenic space of 239 bp with a glycogen phosphorylase homologue immediately upstream (Smith *et al.*, 1996), whereas the operon from *S. sanguis* contains a 365 bp space with an upstream open reading frame aligning with phospho-manno isomerase (Sato *et al.*, 1993). *B. megaterium* and *E. coli* both contain an *atpI* gene and maintain a conserved organizational scheme.

So, we can infer that both loss of the *atpI* gene and re-arrangement of the *atpB* and *atpE* genes in the operon occurred after the Gram-positive and Gram-negative bacteria diverged from one another.

It has been speculated that the large intergenic regions seen upstream of the *S. mutans* (Smith *et al.*, 1996) and *E. hirae* (Shibata *et al.*, 1992) ATPase operons may be involved in the regulation of the operon. Sequence analysis of the putative promoter regions has indicated the presence of inverted DNA repeats (Shibata *et al.*, 1992; Smith *et al.*, 1996). Inverted DNA repeats are becoming established as a feature common in the regulatory schemes in *S. mutans*, though the means by which they effect their function is not understood in detail (Kiska and Macrina, 1994; Shibata and Kuramitsu, 1996; Ajdic and Ferretti, 1998; Chen *et al.*, 1998b). A similar analysis did not yield predicted inverted repeat structures for the *S. sanguis* operon (Kuhnert and Quivey, 1999). However, further examination of the upstream regions revealed that all three streptococci possess unusually high adenosine/thymine content in this area. Future analyses may reveal whether there are conserved motifs beyond the presence of functionally conserved sequences in streptococcal ATPase promoters.

Primer extension analyses have identified guanine residues at position –31 bp and –28, relative to the initial methionine for the *atpE* gene in *S. sanguis* and *S. mutans*, respectively. Using RNA samples prepared from cells growing at steady state at various pH values, it was shown that external pH had no effect on the transcriptional start sites for the operon in either *S. mutans* (Smith *et al.*, 1996) or *S. sanguis* (Kuhnert and Quivey, 1999). Thus, while the inverted repeats in *S. mutans* could be involved in regulation of the operon's transcription, it does not appear that they are part of a scheme that might affect the structure of the mRNA transcript. As noted by others, the *E. coli* mRNA for the operon is unstable, and numerous RNase E sites have been identified within *atpB* (Patel and Dunn, 1995). The apparent stability of the ATPase-specific mRNAs observed during the primer extension experiments with *S. sanguis* RNA suggests that controlled degradation of ATPase message may differ from that seen in enteric bacteria. Identification of the transcriptional start sites permitted an estimation of potential promoter-functional residues for the operons. The results suggested a putative Pribnow box with sequences of TAACT in *S. sanguis* and TAAACT in *S. mutans*, which are similar to the *E. coli* consensus sequence of TATAAT. The –35 bp sequences of TTGACT for *S. sanguis* and TTGACA in *S. mutans* are clearly conserved for the canonical σ^{70} *E. coli* –35 region sequence of TTGACA, suggesting, at least, a house-keeping mechanism for regulating ATPase synthesis.

Since both organisms are apparently capable of up-regulating the amount of ATPase produced in response to acidification, the sequences suggest that the mechanisms are not entirely identical. Previous studies have shown that *S. sanguis*, while not nearly as robust in its response to acidification as *S. mutans*, is

capable of acid-adaptation (Casiano-Colon and Marquis, 1988). If the adaptation in *S. sanguis* includes a global regulatory system, the sequence upstream of the ATPase structural genes shows that nucleotide pattern recognition must differ from the response observed in *S. mutans*.

2.3. Structural and Functional Conservation of Streptococcal F_1 Subunits

The amount of information on the structure of F-ATPases and the functional role of specific residues provides a basis for evaluating differences between the two oral streptococcal forms of the enzyme. *S. mutans* is considered a major etiologic agent for dental caries, owing in some significant amount to the biology of its ATPase. Information regarding the F-ATPase operon of *S. sanguis*, generally considered a commensal organism in the oral cavity, is now available (Kuhnert and Quivey, 1999). Thus, the opportunity exists to evaluate the structural contributions of the F-ATPases to aciduranee of these organisms.

The major amino acid residues known to be involved in catalysis and proton-translocation were conserved, for the most part, in the streptococcal enzymes. These included: the Walker-A sequence (Walker *et al.*, 1982) found in *E. coli* and similar to domains found in other nucleotide binding proteins such as *ras* p21; adenylate cyclase (Walker *et al.*, 1982; Duncan and Senior, 1985; Fry *et al.*, 1986; Garboczi *et al.*, 1988; Duncan and Cross, 1992); and *ercA* (Amano *et al.*, 1994). In *E. coli*, the domain is located at position 149-156 of the *atpD* gene. The Gly-Gly-Ala-Gly-Val-Gly-Lys-Thr sequence is completely conserved in *S. sanguis* and *S. mutans*, though the position is modestly shifted to residues 155-162. Similarly, the nucleotide binding region sequence, Gly-Asp-Arg-Gln-Thr-Gly-Lys-Thr, established for the α subunit (*atpA*), is conserved in both position and sequence, at residues 169-176 in *S. mutans* and *S. sanguis*. The conservation of sequence in the catalytic domains extended well beyond the information in the nucleotide binding clefts, thereby providing additional evidence of the strong pressure in maintaining the ATPase structure in the streptococci. For example, coupling of proton conduction with ATP synthesis has been shown to involve, at least, the interaction of the β380DELSEEDβ386 sequence with the γM23 residue in the *E. coli* F_1F_0 enzyme (ECF_1F_0) (Aggeler and Capaldi, 1996; Al Shawi and Nakamoto, 1997; Ketchum *et al.*, 1998). The γM23 residue was conserved in both of the oral streptococci. The γM23-interactive sequence in the β subunit, though somewhat rearranged as DELSDDE in *S. sanguis* and DELSDEE in *S. mutans* (Smith *et al.*, 1996), still contains information appropriate for interaction with the γ subunit. Residues involved with the rotation of γ-ε have been identified via cysteine-cross-linking mutagenesis (Aggeler and Capaldi, 1996; Aggeler *et al.*, 1997), two of which, αS411/βE381, are conserved in the

S. sanguis and *S. mutans* homologues. Two additional residues implicated in the rotational mechanism, γC87 and εS108, were not conserved in our streptococcal sequences (Aggeler and Capaldi, 1996; Aggeler *et al.*, 1997; Aggeler *et al.*, 1998). Glutamate residues were deduced at position 108 of the ε sequence for *S. mutans* and *S. sanguis*, such that it seems possible the carboxyl-OH might play a role similar to the serine residue in ECF_1F_0. It is difficult to reconcile the absence of cysteine in the γ subunit of both streptococcal forms. However, in both enzymes, position 87 is occupied by serine residues, suggesting the possibility of hydrogen bonding via the hydroxyl group.

Residues shown to be involved with coordination of the magnesium ion in the catalytic site (Weber *et al.*, 1998) were all conserved, including βT156, βE185, and βD242. Residue βE181 is also present even though it has been shown that, despite its reasonable proximity to the metal ion, the residue apparently does not participate in magnesium binding (Weber *et al.*, 1998). A lysine residue at position βK150 of the *S. sanguis* enzyme seems likely to fulfill the site-1 MgATP gamma-phosphate binding role of βK155 in the ECF_1F_0 enzyme (Lobau *et al.*, 1997). The non-catalytic nucleotide binding domains in the alpha subunits of both streptococcal forms retains the conserved aspartate residue at position 261, which is required for MgATP binding but not for catalysis (Weber *et al.*, 1995).

Conservation of functionally important residues extends into the membrane-bound residues as well. NMR data have shown that the *c* subunit (*atpE*) contains two helical regions connected by a polar loop of three amino acids: Arg-41, Gln-42, and Pro-43 (Fillingame *et al.*, 1995). All three of the loop amino acids are conserved in the streptococcal enzymes. The *c*D61 residue in *E. coli* is known to bind the proton involved in ATP-dependent translocation. The residue was substituted in the streptococci as a glutamate residue, similar to that found in *P. modestum* (Esser *et al.*, 1990). The R210 residue in helix-4 of the *a* subunit is known to be physically close to *c*D61 in ECF_1F_0 (Valiyaveetil and Fillingame, 1997; Jiang and Fillingame, 1998) and the residue is conserved in the enzymes from *S. sanguis* and *S. mutans*. Likewise, the highly conserved residue, βR36, which is necessary for retention of coupling (Caviston *et al.*, 1998), has been similarly conserved in the streptococcal forms of the subunit. In the ECF_1F_0 enzyme, *c*Q42 is shielded from *N*-ethylmaleimide labeling by the presence of the γ and ε subunits (Watts and Capaldi, 1997), apparently by association with γY205 (Watts *et al.*, 1996). The *c*Q42 residue has been conserved in *S. sanguis* and a tyrosine residue at position 207 of the γ subunit was recorded. The corresponding position in *S. mutans* is an asparagine (Smith *et al.*, 1996) and its ability to participate in bonding with *c*Q42 is an open question for the present.

It is clear from the strong conservation of relevant amino acids found in the oral streptococci that the mechanisms of function are likely to be identical to

those described thus far for the ECF_1F_0 enzyme. What remains to be determined is the basis for the difference in pH optima for the two enzymes. Previous data have indicated that the kinetic parameters for the ECF_1 enzyme are unaffected by the presence or absence of F_0 (Lobau *et al.*, 1998). Results from studies with F_1 and F_1F_0 preparations from *S. sanguis* and *S. mutans* have shown that pH profiles for F_1 enzyme preparations were more alike than those using membrane-bound or membrane-free preparation of F_1F_0 (Sturr and Marquis, 1992). The conclusion derived from the experiments with the isolated streptococcal enzymes was that the association of F_1 with F_0 led to enhanced acid-tolerance of enzymatic activity. Mixing experiments have not been reported, so it is not yet possible to say that either the F_1 or F_0 domain of the *S. mutans* enzyme confers the pH advantage over the *S. sanguis* enzyme and which residues contribute to the difference.

2.4. Expression of the *S. sanguis* ATPase in *E. coli*

Previous studies with ATPase clones from the Gram-positive organisms, *B. megaterium* (Hawthorne and Brusilow, 1986) and *E. hirae* (Suzuki *et al.*, 1993), indicated that the streptococcal enzymes would also be expressed, functionally, in *E. coli*. Subsequent experiments with a strain defective in the *atpD* gene showed that cloned material from *S. sanguis* did indeed confer on the mutant the ability to hydrolyze ATP (Kuhnert and Quivey, 1999). The constructs used in those experiments did not include the entire operon from *S. sanguis*, indicating the ability of the participating subunits to interact successfully with those produced by *E. coli*. Importantly, the results suggest the possibility that hybrids of *S. mutans* and *S. sanguis* could be constructed in a strain deleted of ATPase subunits in *E. coli*, thereby permitting analyses of pH optima in a well-defined background. DCCD sensitivity of the wild-type *S. sanguis* ATPase indicated a normally functioning enzyme, with ATP cleavage coupled to proton export. However, ATPase activity expressed in the *E. coli atpD* background was DCCD-insensitive, indicating an uncoupled phenotype and the likelihood that, while ATP cleavage was occurring, proton-translocation was probably not (Kuhnert and Quivey, 1999). Expression of the *S. sanguis* clone in the defective *E. coli* strain could have included only seven of the eight structural genes. Therefore, the enzymatic activity could have resulted from any number of possible hybrids of *E. coli/S. sanguis* subunits. We had observed that while the enzyme activity could be measured by ATPase assay, expression of the *S. sanguis* subunits did not provide for growth on minimal succinate medium, supporting the strong likelihood that interaction of the subunits in the hybrid enzymes did not permit coupling of proton pumping to catalysis.

2.5. Regulation of the *S. mutans* F-ATPase Operon

A large number of studies pertaining to the regulation of the F-ATPase in *E. coli* have provided a framework to pursue the question of how the F-ATPase is regulated in the streptococci. The *atp* operon is transcribed as a single mRNA species in *E. coli* of approximately 7 kb, and each gene encodes a single subunit (Jones *et al.*, 1983). One of the primary issues in ATPase biology has been the question of how organisms regulate the stoichiometry of the subunits in the enzyme complex at ratios of $a_1c_{10-12}b_2\delta_1\alpha_3\gamma_1\beta_3\varepsilon_1$ (Foster and Fillingame, 1982). Hypotheses aimed at providing a mechanism for the enzyme's assembly in *E. coli* have included the potential use of secondary structures within the mRNA, which may play a role in the altered subunit expression (Brusilow *et al.*, 1982; Klionsky *et al.*, 1986). Translational coupling has also been proposed as a mechanism of regulation for the operon (McCarthy, 1988; Gerstel and McCarthy, 1989; Hellmuth *et al.*, 1991; Pati *et al.*, 1992; Kravanja *et al.*, 1999). Stability of the *atp* transcript apparently plays a role in the production of enzyme subunits as well. Numerous RNase E sites are present in the transcript, some of which lead to a reduced stability of the 5′ end of the message (Gross, 1991; Patel and Dunn, 1992; Patel and Dunn, 1995). Additional data have linked the stability of the *atpB* gene to the *atpI* mRNA (Hsu and Brusilow, 1995). Finally, overall synthesis of the *E. coli* ATPase operon appears to be growth-rate regulated (Kasimoglu *et al.*, 1996).

The data for the induction of ATPase in oral streptococci was derived from cells growing at a constant growth rate, in a chemostat where pH was held constant at either pH 5.0 or 7.0 (Belli and Marquis, 1991; Hamilton and Buckley, 1991). For that reason, it seems that other mechanisms of regulation must come into play for the oral organisms; though just as clearly, some similarities have been retained. For example, the stoichiometry of the subunits must almost certainly be conserved with the number exhibited by *E. coli*, based on the expression data of the *S. sanguis* enzyme in an *atp*-defective *E. coli* strain. Sequence analysis of the *S. mutans* and *S. sanguis* operons has shown the presence of a large number of potential RNase E sites, which could lend themselves to processing of the ATPase. However, the stability of the 5′ end of the message was clearly sufficient to provide strong primer extension products from these organisms (Smith *et al.*, 1996; Kuhnert and Quivey, 1999). Quite recently, it was shown that the F-ATPase from *L. acidophilus* could be identified by differential display PCR amplification of mRNA in a screen for genes up-regulated during growth at low pH values (Kullen and Klaenhammer, 1999). The results of RNA slot blots indicated that the level of ATPase-specific mRNA was increased, approximately 2- to 3-fold, similar to the increase in enzymatic activity seen in the oral streptococci (Belli and Marquis, 1991).

The mechanism by which transcription of ATPase might be regulated in *S. mutans* or *S. sanguis* is far from clear. However, the presence of inverted DNA

repeats as participants in gene regulation is becoming more widely described for streptococci (Shiroza and Kuramitsu, 1988; Hudson and Curtiss, 1990; Townsend-Lawman and Bleiweis, 1991; Kiska and Macrina, 1994; Shibata and Kuramitsu, 1996; Chen *et al.*, 1998b). The putative inverted DNA repeats found upstream of the *S. mutans* F-ATPase, in place of an *atpI* gene, indicate that there may be an alternative mode of ATPase regulation in *S. mutans*, at least (Smith *et al.*, 1996). The possibility of *cis*-acting elements in the putative inverted repeats upstream of the *S. mutans* ATPase operon has been examined using transcriptional fusions. Portions of the *S. mutans* F-ATPase promoter containing either one and one-half of the two inverted DNA repeats or the proximal one-half repeat were fused to a chloramphenicol acetyl transferase (CAT) gene and expressed from a plasmid carried in *S. mutans* and in *S. sanguis*. Measurement of CAT activity in strains during batch culture growth showed an approximately 2.5-fold increase in CAT activity in the strain carrying the longer fusion compared with strains expressing the fusion with only one-half of the proximal inverted repeat. The data indicated that the region containing both inverted repeats may play a role in the transcription of the ATPase operon (Kuhnert and Quivey, 1999). When the fusions were expressed in steady-state cells, at pH 7.0 and 5.0 in *S. mutans* and at pH 7.0 and 6.0 in *S. sanguis*, the results showed that both fusions provided similar levels of CAT expression; though more activity was seen at pH 5.0 than at pH 7.0. Somewhat surprisingly, the fusions functioned similarly but at approximately half the levels in *S. sanguis*, suggesting that the machinery that operates transcription in *S. mutans* is also present to some extent in *S. sanguis* (Kuhnert and Quivey, 1999). From reported enzyme activity measurements, the up-regulation seen in the CAT activity is in good agreement with the increase in ATPase activity seen when the cells are grown at low pH (Belli and Marquis, 1991; Hamilton and Buckley, 1991). Data from batch-grown cells containing the fusions was somewhat clearer and indicated that the inverted repeats were necessary for optimal expression of the F-ATPase promoter. However, ATPase transcription was apparently supported by as little information as the −10 and −35 region, which was contained within the shorter fusion. The mechanism of regulation by pH appears to be distinct from the regulation imparted by the inverted repeats, due to the fact that the pH regulation is still present in the smaller fusion.

Slot blot analysis of the ATPase-specific mRNA, hybridized to a *atpD*-subunit probe, was in agreement with the levels of CAT activity in the fusion strains (Kuhnert and Quivey, 1999). Taken together, the genetic fusion data and RNA data support the notion that ATPase, in the oral streptococci, is transcriptionally regulated, though at modest levels compared with systems involved with ammonia generation (see below).

A recent report shows that it is very likely that we do not yet fully understand the nature of ATPase synthesis in organisms outside the enteric microorganisms. Study of the regulation of the ATPase in *E. hirae* indicates

that the amount of ATPase is controlled at the level of subunit assembly as a function of pH and that synthesis is not affected by fluctuations in external pH values (Arikado *et al.*, 1999). The data, indicating that unassembled subunits of ATPase are present in the *E. hirae* cytoplasm, is a unique observation and in stark contradistinction to the situation in *E. coli*, where free subunits are not generally found. Data regarding the fate of subunits in oral streptococci await additional experimentation, though the multi-layered regulation of the enzyme's synthesis in *E. coli* should serve to indicate that additional information for the streptococci is likely to be found.

3. PRODUCTION OF BASIC COMPOUNDS BY ORAL STREPTOCOCCI

3.1. Arginine Deiminase System as an Acid-Adaptive Protectant

The problem faced by oral microbes that are not as aciduric as *S. mutans* and the lactobacilli is how to survive in the face of acid stress. Among the mechanisms available to these less acid-resistant organisms is the production of ammonia, which acts to ameliorate local acidic conditions. Indeed, it was observed early on, in studies with human subjects, that the pH of saliva acidified rapidly when the subjects ingested sugar. However, the pH of the saliva would recover over time and gradually become more basic than baseline values (Stephan, 1940, 1944). The apparent reason for the salivary pH recovery was the bacterial production of ammonia from salivary proteins containing either arginine or urea (Stephan, 1944; Wijeyeweera and Kleinberg, 1989a).

Many bacterial strains rely on arginolysis to produce ammonia, as well as ATP (reviewed in Abdelal, 1979; Cunin *et al.*, 1986). In fact, the normally aerobic *Pseudomonas aeruginosa* utilizes the mechanism to support anaerobic growth (Vander Wauven *et al.*, 1984); whereas the lactic acid bacteria carrying out malo-lactate fermentation in wine are also protected by the production of ammonia (Liu and Pilone, 1998). In the present discussion, the system responsible for arginolysis is referred to as the arginine deiminase system (ADS), though it actually consists of a three-enzyme complex including an inducible arginine deiminase, whose function is to liberate ammonia from arginine with citrulline as the product. Ornithine transcarbamylase will then catalyze the condensation of citrulline with inorganic phosphate to produce ornithine and carbamoyl phosphate. Finally, carbamate kinase creates ATP, by the transfer of phosphate to ADP, and the formation of an additional molecule of ammonia.

In most bacteria, the ADS is inducible in the presence of arginine and catabolite repressible in the presence of glucose. Initial observations in

streptococci arose from studies with barotolerant mutant strains of *S. faecium* (Campbell *et al.*, 1985) and *S. sanguis* (Ferro *et al.*, 1983), which were able to degrade both glucose and arginine, suggesting that the catabolite repression had been damaged in the mutants. In the course of those experiments, the organisms revealed a level of acid-tolerance for which a known mechanism was lacking at the time. Subsequent studies were conducted with an expanded list of strains including *S. sanguis*, *S. milleri*, *S. rattus*, as well as *S. faecalis* and *S. faecium* (Marquis *et al.*, 1987; Casiano-Colon and Marquis, 1988), which are commonly isolated from oral sites in humans (Gold *et al.*, 1975). It was found that the ADS functioned below the minimum level of growth for *S. faecalis* (approximately 1.5 pH units) and almost a full pH unit below the minimum glycolytic value (Marquis *et al.*, 1987). Indeed, the system in *S. faecium* was capable of catalysis at a pH value of 2.5. The overall acid-tolerance of the ADS turned out to be true for the expanded list of oral strains as well. A variety of streptococci were shown to form ammonia at initial pH values of 4.0, which was well below the level at which any of the strains are able to grow (Marquis *et al.*, 1987; Casiano-Colon and Marquis, 1988). Moreover, not only was arginolysis functional at low pH values, the arginine formed by the ADS was able to protect cells from acid-mediated killing. Data from acid-survival experiments showed that ammonia formation, at least *in vitro*, had the important physiological trait of protecting cells from acidification (Casiano-Colon and Marquis, 1988), at levels where membrane damage is known to occur (Marquis *et al.*, 1973).

The genes encoding the ADS have been cloned from *S. sanguis* and appear to be grouped in close physical proximity, similar to other systems (Burne *et al.*, 1989a). Using the nomenclature developed for *P. aeruginosa*, *arcA* encodes the AD gene itself, *arcB* encodes the ornithine transcarbamylase, and *arcC* the carbamate kinase. The *S. sanguis* ADS genes were not well expressed in *E. coli*, which is uncommon, in general, for streptococcal genes, leading to speculation that the genes are positively regulated in their native host and not so in *E. coli*. Some evidence does exist for positive regulation in *P. aeruginosa* (Vander Wauven *et al.*, 1984) and in *S. faecalis* (Simon *et al.*, 1982). Interestingly, high levels of expression were reported for the *S. sanguis* ADS expressed in *S. mutans*, suggesting that something in the streptococcal environment was permissive for expression and somehow lacking in *E. coli* (Burne *et al.*, 1989b). Post-translational processing or proximity to membranes might play a role in expression of the ADS in streptococci in general, and supporting this view was the observation that the ADS activity itself was found in greatest proportion in the membrane fraction of *S. sanguis* cell extracts (Burne *et al.*, 1989a). Mutational studies have indicated that *arcA* and *arcB* are likely to be coordinately regulated with *arcC*, though each apparently has its own promoter and *arcC* may be regulated by unknown factors in addition to those regulating *arcAB* (Burne *et al.*, 1989a).

More recently, it has been shown that *S. sanguis* and *S. rattus* can undergo acid-adaptation independently of the induction of the ADS. A triggering pH value in the vicinity of 5.5 resulted in the induction of means to protect these cells from acid-mediated killing (Curran *et al.*, 1995). However, the mechanism behind the ADS-independent protection has yet to be defined. Thus, protection from acid-mediated damage by the ADS appears to be by a mechanism secondary to other elements expressed in *S. sanguis*. Nevertheless, the contribution of the ADS to the cells is important as judged by levels of induction in response to acidification: in *S. rattus*, AD activity was increased 48-fold; in *S. sanguis* 10904, 1467-fold; and in *S. gordonii*, over 300-fold (Curran *et al.*, 1998). These values represent a clear commitment of cellular resources to enzyme production, though it may also reflect the availability of inducer or an inducing mechanism.

The overall importance of the ADS in oral streptococci is to be found in the observation that the ecology of the system is apparently built upon the supply of arginine-containing peptides present in saliva. The difference between caries-free individuals and those who are caries-susceptible appears to be most related to the pH of dental plaque. Studies with human subjects have shown that plaque from caries-free subjects recovers more rapidly from acid formation than does plaque from caries-susceptible persons and to higher, final pH values (Stephan, 1944; Englander *et al.*, 1956; Kleinberg and Jenkins, 1964; Rosen and Weisenstein, 1965; Turtola and Luoma, 1972; Mandel and Zengo, 1973; Abelson and Mandel, 1981; Vratsanos and Mandel, 1982). It was observed some time ago that organisms in dental plaque were capable of forming base (Kleinberg and Jenkins, 1964), which apparently explained the difference between the pH values in plaque fluid recovered from caries-free and caries-active subjects (Margolis and Moreno, 1992). In support of the saliva-based source of base or substrates leading to base were the observations showing that, if salivary contact with dental plaque is blocked, then differences between subjects with and without caries experience becomes insignificant (Abelson and Mandel, 1981). With a potential definition of why some people do not acquire caries while most individuals do, it was of considerable interest to determine the basis for the difference. A number of studies have shown that saliva contains high and low (<3000 M_r) molecular weight proteins and significant levels of free amino acids (Perinpanayagam *et al.*, 1995). The question was whether the source of base was of microbial origin, derived from foods, or formed by the action of proteases on proteins produced in glands and secreted in saliva. Van Wuyckyhuyse *et al.* have shown that samples of ductal saliva, containing no microbial products in otherwise healthy individuals, contain significantly higher levels of arginine and lysine in caries-free subjects than in caries-susceptible subjects (Van Wuyckyhuyse *et al.*, 1995). The results from a parallel study of low-molecular-weight peptides, present in stimulated parotid saliva,

showed that there are indeed arginine and lysine-enriched peptides in the tetra-, penta-, hexa-, and hepta- size ranges (Perinpanayagam *et al.*, 1995). Comparison of sequences indicated the strong likelihood that the small peptides were derived from the salivary histatins (Troxler *et al.*, 1990), acid proline-rich proteins (Hay *et al.*, 1988), basic proline-rich proteins (Kauffman *et al.*, 1991), and statherins (Jensen *et al.*, 1991) via the action of proteases present in the salivary glands or ducts. In addition to the likelihood of host-mediated proteolysis, *S. mitis* (Hiraoka *et al.*, 1986) and *S. sanguis* (Cowman *et al.*, 1983; Rogers *et al.*, 1988) produce arginine aminopeptidases. In the case of the *S. sanguis* aminopeptidase, the enzyme was shown to liberate arginine from short peptides of varying lengths, including tetra-, penta-, and hexapeptides. Peptides containing arginine at the C-terminus were more rapidly processed than those containing arginine at other locations or at the *N*-terminus (Rogers *et al.*, 1988). Two aminopeptidases were reported in *S. mitis*, one of which appeared to be coordinately repressible with the AD system (Hiraoka *et al.*, 1986). Perhaps surprisingly, arginine-containing peptides have been shown to be preferentially utilized over free arginine (Kleinberg *et al.*, 1976; Rogers *et al.*, 1988; Korayem *et al.*, 1990).

3.2. Urease

In addition to arginolysis, a considerable amount of ureolysis is performed also in the mouth, by a variety of bacteria, but most prominently by *S. salivarius*, an organism that is predominant in the dorsum of the tongue (Sissons *et al.*, 1989). The quantities of urea available to oral microorganisms vary, generally between 3 and 10 mM in healthy individuals (Golub *et al.*, 1971; Kopstein and Wrong, 1977) and up to as much as 30 mM in patients with renal disease (Peterson *et al.*, 1985), and 60 mM in gingival crevicular fluid (Golub *et al.*, 1971). Studies conducted early on, and to the present, indicate that urea is metabolized rapidly in the mouth by resident bacteria expressing urease activities. As a result, ureolysis probably contributes a great deal to the ammonia present in dental plaque (Kleinberg, 1967; Biswas and Kleinberg, 1971; Singer *et al.*, 1983; Singer and Kleinberg, 1983a, b; Sissons and Cutress, 1987, 1988; Sissons *et al.*, 1988; Wijeyeweera and Kleinberg, 1989b).

Initial studies on the regulation of ureolysis in batch-grown cultures of *S. salivarius* indicated that urease was expressed constitutively at relatively high levels and was influenced by pH (Sissons *et al.*, 1990). The *S. salivarius* enzyme was shown to have a fairly broad stability to pH with a maximum around 7.0 and retaining approximately 80% of its maximal activity between pH values of 5.0 and 8.5. However, upon exposure to acidic conditions, the enzyme rapidly loses its activity and was essentially zero at pH 4.3. The conclusion is that urease, while clearly able to lift the pH of saliva or dental

plaque to neutral values and above, is not nearly as intrinsically aciduric as the enzymes of the ADS.

Subsequent experiments, with cells growing at steady state, at controlled pH values of 7.0, 6.0, and 5.5, showed that *S. salivarius* would dramatically regulate the amount of urease produced depending on the growth pH, the amount of glucose present in the medium, and the growth rate of the cells (Chen and Burne, 1996). An approximately 1800-fold range in activity was measured between a growth rate of 2.3 hours per generation at a pH value of 7.0 in medium containing 25 mM glucose, and the same growth rate but at a pH value of 5.5 in medium containing 200 mM glucose. The data showed clearly that pH was pre-eminent for control of urease production. However, the amount of available glucose was also a factor in that nearly twice as much urease activity was observed in the presence of 200 mM glucose as compared with 25 mM glucose, at a pH value of 5.5 (Chen and Burne, 1996). The authors noted that the physiological regulatory pattern observed with the urease system was nearly the opposite of that previously reported for the phospho-enol pyruvate dependent-phosphotransferase system (PTS) in *S. salivarius* (Vadeboncoeur *et al.*, 1987, 1991).

Characterization of urease expression in strains defective for $EIIA^{Man}$ was performed to determine the influence of the PTS on the expression of urease. It was already known that glucose, among other sugars, was transported in *S. salivarius* by the action of a two-subunit protein termed $EIIA^{Man}{}_{H}$ and $EIIA^{Man}{}_{L}$ (Bourassa *et al.*, 1990). Removal of the glucose-PTS uptake system resulted in a nearly 20-fold reduction of urease activity (in one of the PTS mutant strains), though the same pattern of pH regulation was observed, indicating that a connection existed between the PTS, sugar metabolism, and the regulation of urease (Chen *et al.*, 1998a). RNA slot blots, probed with a *ureC*-subunit probe, showed that the levels of urease-specific mRNA were tied to pH values, showing that urease is regulated transcriptionally (Chen and Burne, 1996).

Nucleotide sequencing of the cloned urease operon from *S. salivarius* revealed a genetic organization of *ureIABCEFGD*, similar to that from other organisms (Chen *et al.*, 1996). The main catalytic subunits composing the apoenzyme are α, β, and γ, which are encoded by *ureC*, *ureB*, and *ureA*, respectively. The subunits encoded by *ureDEFG* function as accessory proteins and act to assist in the incorporation of nickel ion into the enzyme. The *S. salivarius* urease was expressed and enzymatically active in both *E. coli* and a non-ureolytic oral streptococci, *S. gordonii*. The optimal pH for activity varied modestly from 6.0–7.0 in *S. gordonii* to 7.0 in the *E. coli* background, indicating a more or less native confirmation. Interestingly, the optimal temperature and K_m values were the same for *S. gordonii*, *E. coli* and the native enzyme from *S. salivarius*, at 60°C and 3.7–3.8 mM, respectively. The K_m for the enzyme is in good agreement with the availability of urea in the oral

cavity (3–10 mM). Evaluation of the operon sequence showed the presence of two regions of inverted DNA repeats located 5′ to *ureI*.

Northern blot mapping of urease-specific transcripts indicated two possible promoters for the urease operon, *PureI* and *PureA* (Chen *et al.*, 1998b). *PureI* mapped to σ^{70}-like sequence 22 bases upstream of the start of the *ureI* gene. Expression of urease in streptococcal hosts and in *E. coli*, as well as CAT fusions to a 400 bp region upstream of *ureA*, supported the existence of a functional promoter for the operon. However, the *PureA*–CAT fusions were not responsive to pH; primer extension analysis showed a clear, pH-responsive product for *PureI*, indicating that it functions as the environmentally sensitive promoter element. Immediately upstream of *PureI*, two regions of inverted repeat sequences were identified at positions 26 and 83 bases 5′ of the *PureI* –35 region. CAT reporter constructs, fused either to a complete promoter or to a deletion missing approximately 1.5 of the repeats, were constructed and used to show that both CAT and urease were induced at acidic pH levels (pH 5.5). Further, the fusions were active in the presence of excess carbohydrate, indicating negative regulation of the urease operon (Chen *et al.*, 1998b). Thus, the 100 bases deleted from the *PureI* promoter have a role that integrates regulation by pH and PTS-dependent gene regulation. A model has been presented by these authors consisting of a urease-operon repressor that would be phosphorylated in the absence of sugar and during growth at neutral pH values. When sugar becomes more abundant, the PTS would preferentially phosphorylate sugar, excluding the urease repressor, thereby facilitating *PureI*-driven synthesis (Chen *et al.*, 1998a). What remains to be determined is the nature of the phosphorylation mechanism and the identity of the repressor molecule.

4. LOW pH INDUCTION OF DNA REPAIR

The induction of DNA repair enzymes as part of a global stress response is well-established for enteric bacteria (Farr and Kogoma, 1991). Indeed, the levels of exonuclease III in *E. coli* increase approximately five-fold when the organism is stressed by the presence of hydrogen peroxide (reviewed in Demple and Harrison, 1994). In the course of exploring the physiology of a *recA* strain of *S. mutans* (Quivey and Faustoferri, 1992), the observation was made that adaptation to acidic conditions apparently included a RecA-independent DNA repair system. The nature of the system was such that growth of *S. mutans* at pH values near 5.0 resulted in enhanced levels of resistance to acid treatment, UV-B irradiation, and exposure to hydrogen peroxide, similar to acid-adapted wild-type strains (Quivey *et al.*, 1995). The result suggests that RecA was relatively dispensable at low pH owing to the presence of a DNA repair activity. The task then becomes one of identifying elements of the

inducible repair system that could cope with the types of damage that might arise from multiple damaging agents. The enzymatic capability of removing a wide variety of damaged DNA bases had been established in *E. coli* and many other organisms, including humans (reviewed in Demple and Harrison, 1994; Wallace, 1998). With that background in mind, experiments were designed to determine whether the observations regarding the inducible activity were attributable to a nuclease, such as exonuclease III of *E. coli*. Initial experiments were performed using a double-stranded DNA substrate containing tetrahydrofuran as a model abasic site residue. Subsequent results showed that extracts prepared from cells grown at steady state at pH 5.0 contained nearly five-fold more enzymatic activity than extracts prepared from cells grown at pH 7.0 (Hahn, 1999). Additional studies showed that the inducible activity was unable to recognize substrates containing deoxy-adenosine, a substrate for endonuclease IV, an inducible enzyme in *E. coli* (Ide *et al.*, 1994). Taken together, the data indicated that the inducible system contained, at least, an enzyme with properties similar to the exonuclease III protein from *E. coli* and probably unlike the endonuclease IV (Hahn *et al.*, 1999).

Exonuclease III is a member of a growing group of DNA repair enzymes, referred to as class II AP endonucleases (Demple and Harrison, 1994; Wallace, 1998). The majority of apurinic/apyrimidinic (AP) endonucleases that have been characterized are multi-talented enzymes, displaying a variety of activities. The mode of substrate recognition has become an extremely interesting point of discussion, which was probably initiated by Weiss, in 1976, in an effort to account for the diverse actions of the single-subunit *E. coli* enzyme, exonuclease III (Weiss, 1976).

To strengthen the hypothesis that the Smn enzyme of *S. mutans* falls into this group of multi-functional proteins, and to elucidate its biochemical characteristics, we examined the Smn protein for additional functions, namely 3′ to 5′ exonuclease and RNase H activities. It turned out that the Smn protein and *E. coli* exonuclease III are very comparable in their 3′ to 5′ nuclease activities. The product formation pattern created by Smn, acting on a duplex DNA structure, showed an accumulation of a couple of product bands that were a few nucleotides longer than the predominant bands observed in the Exo III cleavage pattern (Hahn, 1999). Based on the data from the Smn cleavage patterns, it could be argued that Smn dissociates from the substrate sooner than does exonuclease III. In other words, Smn perhaps cleaves off fewer mononucleotides per binding of enzyme to substrate compared with exonuclease III. However, at this point, experimental evidence is lacking to support this idea and the enzyme-substrate dissociation may be purely sequence-dependent.

Sequence-dependency may be a factor for the observed ribonuclease activity of Smn, which certainly seems to be endonucleolytic in nature. The observed cleavage pattern by Smn of an RNA–DNA hybrid structure was very

similar, if not identical, to the pattern created by *E. coli* ribonuclease H (Hahn, 1999). RNase H activities can be detected in all organisms from bacteria to mammals. Nonetheless, the biological role of cellular RNase H is not all that clear. However, based on studies in *E. coli*, some functions for RNase H activities in bacterial cells have been proposed. These include participation in removing RNA primers from Okazaki fragments in discontinuous DNA synthesis (Ogawa *et al.*, 1984) and processing of RNA transcripts to be used as primers for DNA polymerase I as part of the replication cycle of ColE1-type plasmids (Itoh and Tomizawa, 1980).

All class II AP endonucleases have a common endonucleolytic reaction mechanism for cleavage of the sugar–phosphate backbone of the DNA at the site of base loss. These enzymes cleave the phosphodiester bond immediately 5′ of an AP site, producing a 3′-hydroxyl group and a 5′ abasic residue (Demple and Harrison, 1994). To show that the *S. mutans* enzyme also shares this reaction mechanism, we demonstrated that the 3′ end produced by AP endonucleolytic cleavage by Smn was extended by T7 Sequenase, an enzyme that requires a 3′-hydroxyl group for the incorporation of nucleotides (Hahn, 1999). Based on the genetic and functional analysis of the *S. mutans* Smn protein, it seems clear that the Smn enzyme should be considered a member of the class II AP endonucleases.

Besides the questions regarding the role of the Smn nuclease in the acid-adaptive physiology of *S. mutans*, questions regarding the basic biology of the enzyme itself still exist. Recent work has shown that the *S. mutans* Smn protein is a multi-functional enzyme, similar but not identical to the known class II AP endonucleases described by many others. Structural studies of Smn and other AP endonucleases should help to identify the features shared by these enzymes, which allows them to carry out such a multitude of reactions. Some time ago, Weiss proposed a model to explain these seemingly dissimilar activities displayed by this class of enzymes. He called it the 'unifying common site' model, whereby the enzyme recognizes a space created by a missing or a displaced base that exists in all substrates. The model would also hold true for the exonuclease activity. Weiss hypothesized that the fraying which occurs at the terminus of a duplex DNA strand looks just like a missing base (Weiss, 1976, 1981). It is therefore not surprising that single-stranded DNA or RNA do not provide a substrate for the exo- and endonucleolytic activities of Smn.

It would also be of interest to investigate whether the Smn protein has 3′-repair diesterase activity as many of the class II enzymes have (Demple and Harrison, 1994). A characteristic form of oxidative DNA damage is free radical-initiated breaks bearing 3′-phosphates or 3′-phosphoglycolate esters (Henner *et al.*, 1983; Demple *et al.*, 1986). The ability to recognize and repair such 3′ blocking residues would implicate Smn directly in the repair of oxidative damage lesions. In fact, recent data seem to suggest already that an *S. mutans smn* mutant strain is more sensitive to the oxidative damage inducing

agent H_2O_2 than a *S. mutans* wild-type strain (R.G. Quivey, Jr, unpublished data). Studies of all the *E. coli* exonuclease III mutants have shown that the exonuclease, phosphatase and endonuclease activities are similarly affected (Weiss, 1981). It is also of great interest to determine whether Smn can substitute for the function of exonuclease III in an *E. coli xth* strain, or whether the function of other class II AP endonucleases can be complemented by *S. mutans* Smn. If complete complementation is not observed, then perhaps other *S. mutans* enzymes may be involved in the repair of those DNA lesions.

Availability of the deduced amino acid sequences for the *E. coli* and *S. pneumoniae* exonuclease genes permitted the identification of two regions of conserved sequence. Using the conserved domains, polymerase chain reaction was used to amplify homologous material from *S. mutans*, which was subsequently used as a probe to identify a genomic clone encoding the Smn protein. The deduced amino acid sequence of the gene was highly homologous to the enzyme from *S. pneumoniae* (82% identity) (Martinez *et al.*, 1986; Puyet *et al.*, 1989) and as expected, considerably less to the *E. coli* enzyme (Rogers and Weiss, 1980; Hahn, 1999; Hahn and Quivey, unpublished). The molecular mass of Smn was estimated at approximately 33 kDa for its 276 amino acids, which is comparable to the size of several of the class II AP endonucleases. Further, the results of protein modeling of the *S. mutans* enzyme showed strong structural similarity to the structure derived from 1.7 Å X-ray crystallographic data for the *E .coli* enzyme (Mol *et al.*, 1995). Conservation of the active site and metal binding residues (Asp-229, His-259, and Glu-34 in *E. coli*) suggest that the *S. mutans* Smn protein may also have significant functional homology.

Promoter analysis of the *smn* 5′ untranslated region and determination of the transcriptional start site revealed that the gene is transcribed as a monocistronic mRNA and not as part of a polycistronic message. The *smn* promoter structure showed a high degree of identity to the *E. coli* consensus sequences of promoters (Hahn, 1999). A more detailed study of the *smn* promoter region is clearly desirable given that the expression of the enzyme appears to be induced by low pH conditions. A key issue is that in *S. mutans*, at least, an exonuclease homologue was induced by growth at low pH. Typically, the *xth* gene, encoding the *E. coli* exonuclease III, is induced by the presence of oxidative damaging agents, particularly hydrogen peroxide (Demple *et al.*, 1983). For that reason, it is of considerable interest to investigate whether there are additional agents or growth conditions that up-regulate the expression of *smn*. Data of this sort would provide needed insights into whether the enzyme is synthesized in response to stressors, in general, or whether, in the lower pH_i environment of the streptococci, acid-mediated damage of DNA mimics oxidative damage. Results from these kinds of studies will help in our understanding of how *S. mutans* regulates its repertoire of acid-inducible proteins.

Insertional inactivation of *smn* in *S. mutans* resulted in the nearly complete loss of AP endonuclease activity, confirming that the *smn* gene does indeed code for a protein with AP endonucleolytic activity, and that Smn is the major AP endonuclease of *S. mutans*. This does not rule out, of course, the possibility that another such enzyme is present in *S. mutans*. The physiological characterization of not only the *S. mutans smn* mutant strain but also of a *recA smn* double mutant appears critical for answering questions regarding the role of the AP endonuclease as part of the acid-adaptation process in *S. mutans*.

5. ROLE OF THE MEMBRANE IN ACID SENSITIVITY

With the amount of information available to indicate that the streptococcal membrane provides an important barrier function for protection from acid, it is therefore perhaps not surprising that acid-sensitive mutations in proteins participating in membrane synthesis and assembly have been identified. For example, Tn*917* mutagenesis has been successfully used to create and screen for acid-sensitive mutant strains of *S. mutans*. The insertions were cloned by marker-rescue and the nucleotide sequence of one of them indicated strong homology to the *B. subtilis ylmX-ffh* locus (Gutierrez *et al.*, 1996). YlmX presently has no known function. However, Ffh is a homologue of the mammalian 54 kDa signal recognition particle and is involved with protein secretion (reviewed in Luirink and Dobberstein, 1994). The *ffh* mutant strain was later shown to produce reduced amounts of F-ATPase, was unable to ferment sorbitol, and apparently was unable to grow at wild-type rates at pH values of 5.0 (Gutierrez *et al.*, 1999). The results indicate a role compatible with membrane assembly and the difficulty cells would have with stress if proteins with a synthetic function become disabled. RNA slot blot results indicated the likelihood that transcription of *ffh* was up-regulated 4- to 8-fold in wild-type cells in response to acid-shock at pH 5.0 (Gutierrez *et al.*, 1999). Clearly, it will be of interest to determine whether Ffh plays a role in the assembly of proteins with a known interaction in membranes, such as the F-ATPase. Indeed, existing data point to the assembly of ATPase as a key regulatory mechanism for the enzyme in response to acidification in *E. hirae* (Arikado *et al.*, 1999).

One example already exists for the role of a synthetic protein in the aciduance of *S. mutans*. Tn*916* mutagenesis of *S. mutans* strain GS-5 has led to the recovery of a chromosomal fragment containing coding regions for proteins with strong homology to diacylglycerol kinase (*dagK*) and Era, a protein with strongly conserved sequences among bacteria and eukaryotes (Yamashita *et al.*, 1993b). DagK normally functions in fatty acid synthesis, though it is thought to play a comparatively minor role in the synthesis of phosphatidic

acid in *E. coli*. However, the level of its participation in lipid synthesis in *S. mutans* is not yet understood and there is literature to indicate a potential role in signal transduction in *E. coli* (Walsh *et al.*, 1986, 1990). Consistent with a multiple role for *dagK* in the protection of *S. mutans*, Yamashita *et al.* reported that the strains carrying the original Tn*916* insertion were not only acid-sensitive, but were also altered in their sensitivities to osmotic pressure exerted by NaCl and to temperature (Yamashita *et al.*, 1993b). The function of Era in other bacteria is not entirely understood, though the protein contains a highly conserved GTPase domain and the enzyme is essential for bacterial growth. Recently, it has been shown that the Era G-protein also contains an RNA-binding domain which is required for Era to function properly (Johnstone *et al.*, 1999). Johnstone *et al.* have speculated that the GTPase binding domain may regulate RNA binding in response to unknown cellular stimuli (Johnstone *et al.*, 1999), pointing out that it has been shown that *E. coli* Era interacts with nucleoside diphosphate kinase, apparently from both *E. coli* and *Pseudomonas aeruginosa*. Because Era associates with the inner membrane of *E. coli*, the authors suggest the possibility that the function of Era might be to interface energy metabolism with macromolecular synthesis at the level of the ribosome (Johnstone *et al.*, 1999).

6. ADAPTATION OF SUGAR METABOLISM

A discussion of the acid-adaptive abilities of the oral streptococci must also include mention of the ways in which these highly saccharolytic organisms metabolize sugar. Because the human diet tends to provide sugars at spaced intervals, the organisms are faced with the prospect of extended periods without significant supply of carbohydrates. The PEP-dependent phosphotransferase system (PTS) acts to regulate the uptake of many monosaccharides for the oral streptococci (Vadeboncoeur and Pelletier, 1997). During periods when sugar is relatively unavailable, the PTS acts as a high-efficiency mechanism to scavenge carbohydrate nutrients. However, when sugar is relatively abundant, the organisms metabolize it rapidly to acidic end-products, resulting in a rapid decrease in external pH. At a pH value of 5.0, *S. mutans* strain Ingbritt was found to be devoid of EII, the membrane component of the PTS that acts to enzyme that phosphorylates glucose as it is taken up into the cell. Thus, pH appears to have a strong effect on the transcription of a protein that directly participates in sugar metabolism. Down-regulation of EII synthesis also affects the uptake of mannose, 2-deoxyglucose, and fructose (Vadeboncoeur *et al.*, 1991). Interestingly, the EII specific for fructose was highest at pH 5.5 and high growth rates (Rodrigue *et al.*, 1988). The mechanisms that regulate EII are still being investigated, though recent data indicate that the protein referred to

as $IIAB^{Man}$, and the phosphocarrier protein HPr, are key regulatory components for the PTS system (Vadeboncoeur and Pelletier, 1997). The question then arises as to how the cell takes up sugar at low pH values. The versatile streptococci express a high-affinity, PTS-independent glucose permease that functions at low pH to take up glucose (Cvitkovitch *et al.*, 1995). The nature of the permease and its characteristics, including potential regulatory circuits controlling its synthesis, remain to be elucidated.

Most of the oral streptococci produce and secrete one or more extracellular polymerases known as glucosyltransferases (GTFs) and fructosyltransferases (FTFs) (Kuramitsu *et al.*, 1995). The GTFs and FTFs act to form polymers of either glucose or fructose. Loss of the enzymes greatly reduces the virulence of *S. mutans* in the rat model for dental caries (Munro *et al.*, 1993; Yamashita *et al.*, 1993a), such that interest in the production and regulation of these important enzymes remains very high. Genetic fusions of the CAT-reporter system have been used to show that transcription of the *gtf* and *ftf* genes is complex, including sensitivities to available carbohydrate, growth rate, and external pH (Hudson and Curtiss, 1990; Wexler *et al.*, 1993). The most surprising observation from these experiments was that extracellular FTF activity did not correlate well with transcriptional activity measured by CAT assay (Wexler *et al.*, 1993). Whereas extracts prepared from cells grown at pH 6.0 contained more than twice the CAT activity than those prepared from cells grown at pH 7.0, the extracellular polymer synthesis was actually several-fold lower. While it was clear that external pH value influenced transcription of *ftf*, the mechanism to explain the differences in external activity remains somewhat unclear. Active processing and release from the *S. mutans* cell surface of proteins, including glucosyltransferase, has been described previously (Lee, 1992), suggesting that the complete picture for GTF and FTF production will come into focus when the post-transcriptional mechanics for these enzymes is more complete.

7. CONCLUDING REMARKS

Here, we have attempted to provide the reader with an update on the progress that is being made in understanding the mechanisms oral streptococci employ to survive acidic conditions in the human mouth. The most clearly pathogenic organism of the group, *S. mutans*, has drawn the greatest share of experimental attention and that is surely reflected in the comments. However, several additional points should be included. Reports have also been made suggesting that perhaps many strains of aciduric, non-mutans strains of streptococci exist, which may also participate in the disease process (Van Houte *et al.*, 1991a, b). Not only is our present understanding of the biology of *S. mutans* incomplete,

but our knowledge of the number and kinds of bacteria that explain oral disease is also incomplete.

By necessity, we have tried to limit the discussion in this article to those systems from which interpretable data are now widely available. While multiple systems for acid-resistance have been described for the enteric bacteria (see, for example, Castanie-Cornet *et al.*, 1999), it is not yet clear how many systems may exist for oral streptococci. However, as the genomes of these organisms become complete (for example, Roe *et al.*, 1999) and experiments designed to yield proteomic maps become firmly established (for preliminary reports see Svensäter and Hamilton (1999) and Cvitokovitch *et al.* (1999)), we will begin to understand how many proteins in a given streptococcus are under the control of pH or generalized stress-response regulation.

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